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Discovery of pyrrolo[3,2-*c*]quinoline-4-one derivatives as novel hedgehog signaling inhibitors

Tomohiro Ohashi *, Yuya Oguro, Toshio Tanaka, Zenyu Shiokawa, Sachio Shibata, Yoshihiko Sato, Hiroko Yamakawa, Harumi Hattori, Yukiko Yamamoto, Shigeru Kondo, Maki Miyamoto, Hideaki Tojo, Atsuo Baba, Satoshi Sasaki

Pharmaceutical Research Division, Takeda Pharmaceutical Company Limited, 26-1, Muraoka-higashi 2-chome, Fujisawa, Kanagawa 251-8555, Japan

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ABSTRACT

The Hedgehog (Hh) signaling pathway plays a significant role in the regulation of cell growth and differentiation during embryonic development. Since activation of the Hh signaling pathway is implicated in several types of human cancers, inhibitors of this pathway could be promising anticancer agents. Using high throughput screening, thieno[3,2-*c*]quinoline-4-one derivative **9a** was identified as a compound of interest with potent in vitro activity but poor metabolic stability. Our efforts focused on enhancement of in vitro inhibitory activity and metabolic stability, including core ring conversion and side chain optimization. This led to the discovery of pyrrolo[3,2-*c*]quinoline-4-one derivative **12b**, which has a structure distinct from previously reported Hh signaling inhibitors. Compound **12b** suppressed stromal Gli1 mRNA expression in a murine model and demonstrated antitumor activity in a murine medulloblastoma allograft model.

1. Introduction

The hedgehog (Hh) signaling pathway plays an important role in cell proliferation and control of survival signals[1] in tumorigenesis as well as in embryogenesis. The mechanism of Hh signal activation has been elucidated. Three Hh family ligands, Sonic hedgehog (Shh), Desert hedgehog (Dhh) and Indian hedgehog (Ihh), bind to the twelve-transmembrane receptor protein Patched (Ptch), which suppresses the activation of the seven-transmembrane signal component protein Smoothened (Smo).[2] The activated Smo upregulates Gli transcription activity, and subsequently, genes under the control of Gli activate downstream signals that contribute to cellular proliferation and differentiation. This signaling pathway is inactive in most adult tissues; however, aberrant activation driven by Ptch-defective mutations or Hh-ligand overexpression have been detected in certain types of cancers.[3] In the mutation-driven mechanism, tumor growth is enhanced by autonomous activation of Gli caused by constitutive Smo activation due to loss of Ptch function. This mechanism has been observed in medulloblastoma[4] and basal cell carcinoma (BCC).[5] In the Hh-ligand driven mechanism, the overexpression of Hh ligand in cancer cells leads to abnormal activation of Gli in neighboring stromal cells, and induces the enhancement of tumor growth by a paracrine mechanism.[6] This mechanism has been detected in a wide range of solid tumors including colon,[7] prostate,[8] and pancreatic cancers.[9] Based on these results, it has been suggested that an Hh signaling inhibitor may be useful as a therapeutic agent for these cancers.

To date, several small molecule Hh signaling inhibitors have been identified (Fig. 1). Cyclopamine, a steroid jerveratum alkaloid derived from corn lily, showed significant activity in Hh signaling pathway inhibition[10] and directly interacts with Smo protein, thereby inhibiting tumor growth in several cancer cell types. The synthetic small molecule Hh signaling inhibitor, vismodegib[11] has been approved by FDA in advanced BCC. Other compounds in development include a cyclopamine-derivative, saridegib (IPI-926),[12] a synthetic compound, NVP-LDE225,[13] and small-molecule inhibitor PF-04449913.[14]

Several compounds of interest, including thieno[3,2-*c*]quinoline-4-one **9a,** were identified as Hh signaling inhibitors by high throughput screening (HTS) in a Gli-luc reporter assay. The chemical structure of **9a** is distinct from those of known Hh signaling inhibitors currently in clinical trials (Figs. 1 and 2). Compound **9a** demonstrated potent inhibition of Hh activity with an IC_{50} of 5.1 nM in the Gli-luc reporter assay; however **9a** was unstable in mouse hepatic microsomes. In the metabolite analysis, the oxidation of the amide moiety was observed in mouse hepatic microsome, thus we conducted chemical modifications of **9a** focused on amide moiety in an effort to enhance metabolic stability while maintaining in vitro activity. In this report, we describe synthesis and SAR of this new class of Hh signaling inhibitors.

* Corresponding author. Tel.: +81 466 32 1151; fax: +81 466 29 4448.
E-mail address: tomohiro.oohashi@takeda.com (T. Ohashi).

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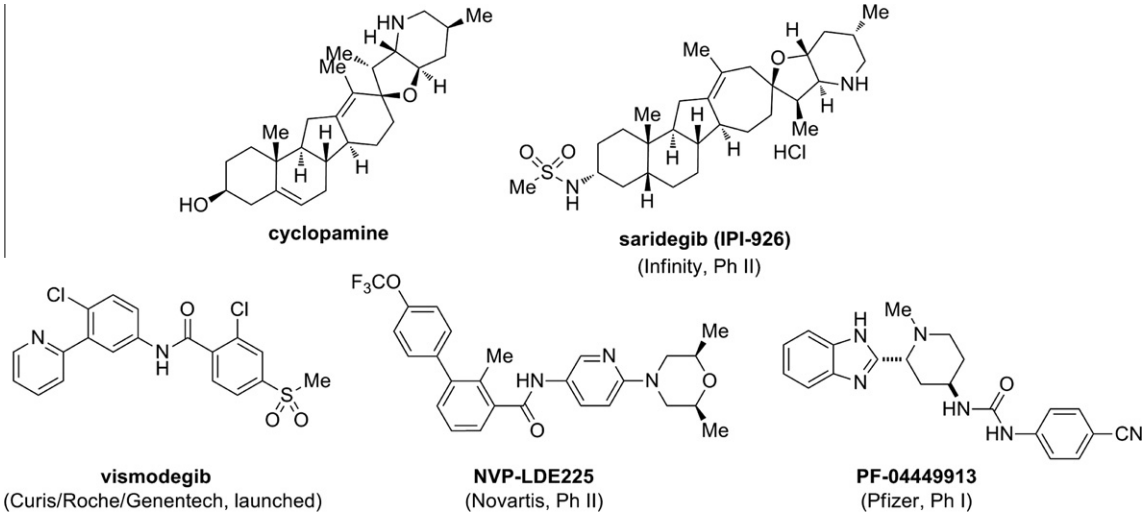


Figure 1. Representative Hh signaling inhibitors.

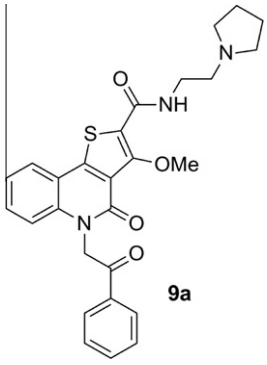


Figure 2. Structure of compound **9a**.

2. Chemistry

Thieno-, pyrrolo-, furo-[3,2-*c*]quinoline-4-one derivatives were prepared as shown in Scheme 1 and Scheme 2. Treatment of methyl anthranilate **1** with diethyl malonate under basic conditions gave quinoline compound **2**. Chlorination of **2** using phosphoryl chloride was achieved in 79% yield. Hydrolysis of **3** with sodium acetate led to 4-chloroquinoline **4** selectively, which was N-alkylated with phenacyl bromide or *p*-methoxybenzyl chloride to afford **5a, b**. The tricyclic core ring was constructed by treatment of **5** with corresponding ester reagent under basic conditions. Alkylation of hydroxyl group of **6** afforded alkoxy derivative **7**, and following hydrolysis gave derivative **8**. Carboxylic acid **8** was converted to various amides **(9a–h)** by condensation with corresponding amines, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt) in 18–93% yield. Piperidine derivative **9i** was afforded by removal of the Boc group of **9h** with HCl (Scheme 1).

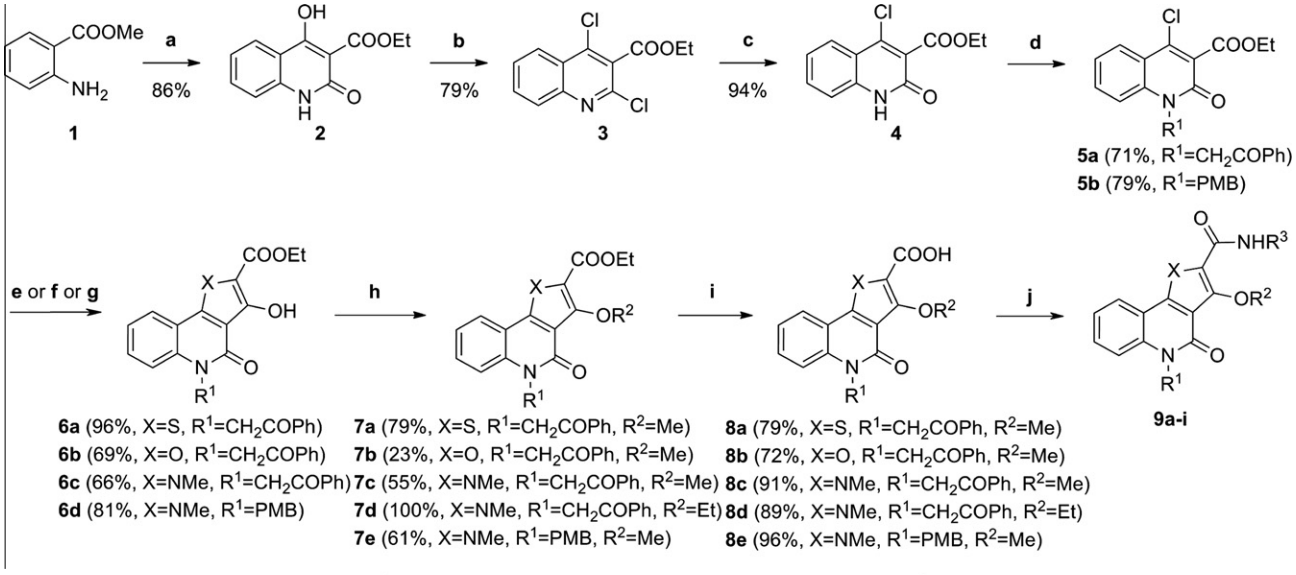
N-Hydroxyethyl **12a** and *N*-hydroxyacetyl **12b** derivatives were obtained from **9g** by its alkylation or acylation in 33% and 51% yield, respectively. **9i** was alkylated with 2-bromoethanol to afford **10**, and the removal of the *p*-methoxybenzyl group was carried out with trifluoroacetic acid (TFA), anisole, and trifluoromethanesulfonic acid (TfOH) at 80 °C, followed by acetylation to give **11**. N-alkylation of **11** using the corresponding alkyl halides in the presence of sodium *tert*-butoxide (*tert*-BuONa) followed by hydrolysis of the acetyl group gave **12c, d** in 15% and 2.4% yield, respectively (Scheme 2).

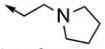
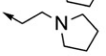
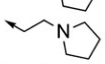
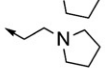
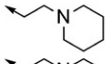
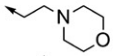
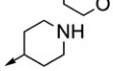
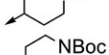
3. Results and discussion

We initially assessed the in vitro effects of the tricyclic core on activity (Table 1) using a luciferase reporter in NIH3T3 cells carrying a stably transfected Gli-reporter construct (Gli-luc reporter cell line).[15] The furo analog **9b** clearly had decreased Hh inhibitory activity compared with **9a**. On the other hand, a significant increase in Hh inhibition was observed with N-methylpyrrolo analog **9c** compared to **9b**. We considered the *N*-methyl moiety of the 5-membered ring to be essential for potent activity, and thus selected *N*-methylpyrrolo[3,2-*c*]quinoline-4-one as a core ring system for further investigation.

Although **9c** showed potent in vitro activity as mentioned above, the metabolic stability of this compound assessed by incubation in mouse hepatic microsomes was still insufficient to suggest that **9c** would have in vivo efficacy in mice (Table 2). Thus, our interest was shifted to improvement of the metabolic stability by optimization of the substituents at the 2, 3, 5-positions of the pyrrolo[3,2-*c*]quinoline-4-one.

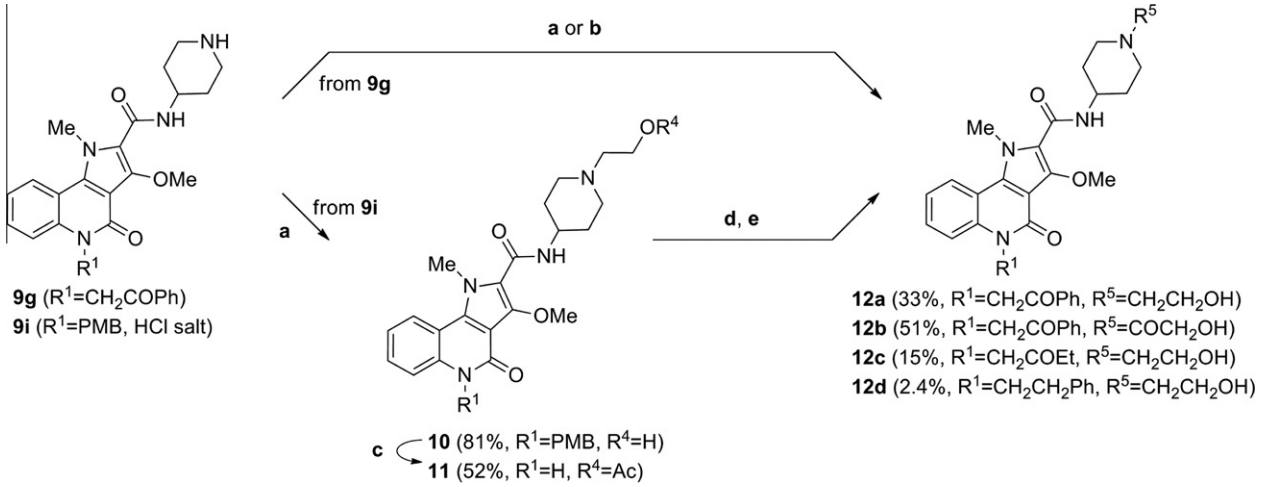
Substitution of methoxy group at the 3-position reduced activity and was apparently unstable in mouse hepatic microsomes (**9d** vs **9c**). Therefore, we fixed the substituent at this position as methoxy in the remaining studies. Several derivatives were prepared with various cyclic amines at the terminal of the side chain at the 2-position. Ethyl groups with piperidine (**9e**) or morpholine (**9f**) derivatives resulted in decreased Gli-luc reporter activity without improvement of metabolic stability. The 4-piperidinyl derivative **9g** showed dramatic improvement in metabolic stability compared to **9c**, indicating that the cyclic amine component attached directly to the amide linker decreased lipophilicity and improved metabolic stability. The side chain of **9g** is shorter than that of **9c** and we speculated that introduction of the ethyl group on to the nitrogen of the piperidine ring in **9g** was responsible for enhancing activity. The overlays of the stable conformation of **9g** and ethylated **9g** (Et-**9g**) calculated by Molecular Operating Environment (MOE) software were shown in Figure 3. It demonstrated that the ethyl group of Et-**9g** could occupy the same space as the pyrrolidine ring of **9c**. The lipophilicity of Et-**9g** was predicted to be higher than that of **9g** (*c*Log*P*: Et-**9g** = 3.5 vs **9g** = 2.5), suggesting that Et-**9g** may have decreased microsomal stability as a result. Thus we introduced a hydroxyethyl substituent (*c*log*P* value: 2.2) onto the piperidine nitrogen of **9g** instead of the ethyl group position. As expected, **12a** demonstrated potent Gli-luc reporter activity while retaining metabolic stability both in mouse and human



cmpds.	X	R^1	R^2	R^3	yield (%)
9a	S	CH_2COPh	Me		62
9b	O	CH_2COPh	Me		66
9c	NMe	CH_2COPh	Me		78
9d	NMe	CH_2COPh	Et		18
9e	NMe	CH_2COPh	Me		90
9f	NMe	CH_2COPh	Me		82
9g	NMe	CH_2COPh	Me		85
9h	NMe	PMB	Me		93
k					
9i	NMe	PMB	Me		99[1)]

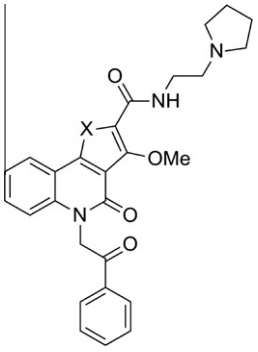
1) yield from **9h**

Scheme 1. Reagents and conditions: (a) diethyl malonate, NaOEt, EtOH, rt then 140 °C; (b) $POCl_3$, 110 °C; (c) AcONa, AcOH, 120 °C; (d) $PhCOCH_2Br$, NaH, DMF, rt for **5a**, or PMBCl, NaH, DMF, rt for **5b**; (e) $EtOOCCH_2SH$ for **6a** or $EtOOCCH_2OH$ for **6b**, NaOEt, EtOH, rt; (f) (1) $MeNHCH_2COOEt{\cdot}HCl$, Et_3N, EtOH, 85 °C; (2) NaOEt, EtOH, 50 °C for **6c**; (g) Et_3N, EtOH, 85 °C then NaOEt, EtOH, 60 °C for **6d**; (h) MeI or EtI, DBU, DMF, rt; (i) NaOH, EtOH, H_2O, rt; (j) R^3NH_2, EDC, HOBt, Et_3N, DMF; (k) HCl, AcOEt, rt.



Scheme 2. Reagents and conditions: (a) 2-bromoethanol, K_2CO_3, DMF, 80 °C for **10**, 100 °C for **12a**; (b) (1) $ClCOCH_2OAc$, Et_3N, THF, 0 °C, (2) NaOH, EtOH, rt; (c) (1) TFA, anisole, TfOH, 80 °C; (2) AcCl, Et_3N, THF, 0 °C; (d) $EtCOCH_2Br$ or $PhCH_2CH_2Br$, *tert*-BuONa, LiBr, DME/DMF, 0 °C to rt. (e) NaOH, EtOH, H_2O, rt.

Table 1
Effect of tricyclic ring core in the Gli-luc reporter assay



Compds	X	Gli-luc reporter IC_{50} (nM)[a]
9a	S	5.1
9b	O	>1000
9c	NMe	2.9

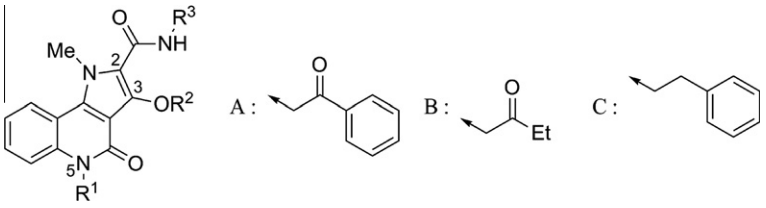
[a] IC_{50} values represent the mean of four measurements.

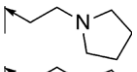
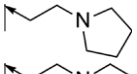
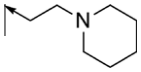
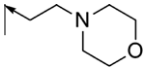
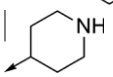
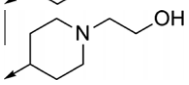
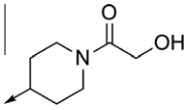
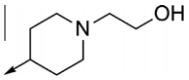
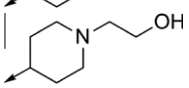
microsomes. However, further evaluation of **12a** showed strong inhibition of the human ether-a-go-go related gene (hERG; data not shown) which is involved in cardiac repolarisation. Since basicity of compounds is often correlated with hERG inhibition,[16] the acyl function was introduced to decrease basicity. Compound **12b**, bearing a hydroxyacetylpiperidine moiety, achieved both potent activity and good metabolic stability without hERG inhibition, as expected.

Finally, the SAR around the 5-position was investigated. The 2-butanoyl derivative **12c,** which had an ethyl group in place of the benzene ring in **12b,** exhibited significantly decreased potency. The phenethyl derivative **12d** without the carbonyl group showed 30-fold drop in activity compared with **12a**. These results suggested that an aromatic substituent at the 5-position would be necessary for tight binding to the Smo protein. Therefore, the phenacyl group in **12b** was selected as the best substituent at the 5-position for further evaluation.

The in vivo pharmacodynamic and pharmacokinetic profiles of **12b** are shown in Table 3. PAN-04 is a human pancreatic xenograft tumor line derived from a clinical specimen established by the Central Institute for Experimental Animals (Kanagawa, Japan). This xenograft tumor expressed significant stroma-derived Gli1 and cancer-derived Shh activity. We measured the reduction of Gli1 mRNA expression levels as a pharmacodynamic marker in this model. Compound **12b** showed potent activity in a Smo binding assay (IC_{50}: 41 nM), favorable in vitro metabolic stability in both mouse and human microsomes, and a favorable mouse

Table 2
Effect of substituents on pyrrolo[3,2-*c*]quinoline-4-one at 2, 3, and 5-position



Compds	R^1	R^2	R^3	Gli-luc reporter IC_{50} (nM)[a]	In vitro clearance[b] (μL/min/mg)	
					Mouse	Human
9c	A	Me		2.9	95	14
9d	A	Et		7.0	155	26
9e	A	Me		9.6	192	30
9f	A	Me		22	237	73
9g	A	Me		21	10	2
12a	A	Me		6.6	9	6
12b	A	Me		4.6	11	<1
12c	B	Me		>100	<1	3
12d	C	Me		150	48	72

[a] IC_{50} values are the mean of four measurements.
[b] Hepatic microsomes.

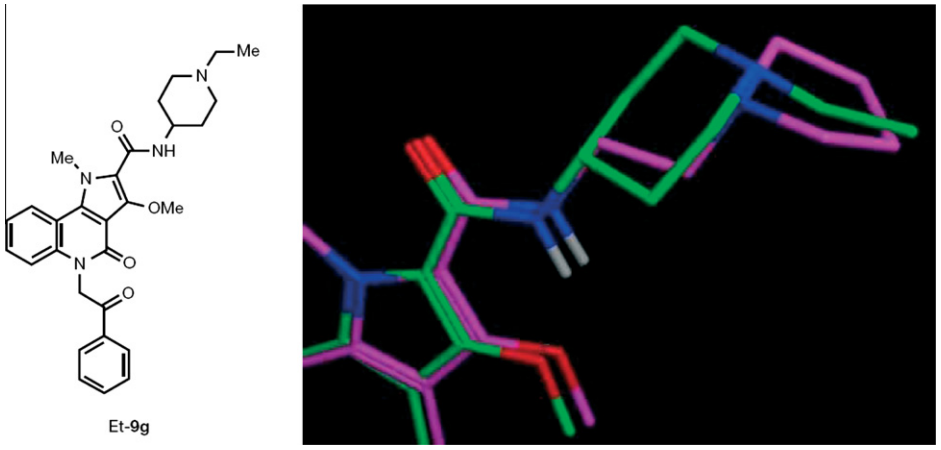


Figure 3. Overlay of Et-**9g** (green), and **9c** (purple) in stable conformation.

Table 3
Pharmacokinetic and pharmacodynamic characteristics of compound **12b**

Compd	Smo binding (nM)	In vivo PD Gli mRNA (% of ctrl)[a]	In vitro clearance[b] (μL/min/mg)		Mouse PK[c]				
			Mouse	Human	C_{max} (μg/ml)	T_{max} (h)	MRT (h)	AUC (μgh/mL)	C_{8h} (h)
12b	41	5	11	<1	2.65	1.00	2.99	12.1	0.604

[a] Gli1 mRNA expression at 25 mg/kg BID, 24 h post-dose. The value indicates Gli1 mRNA expression levels compared to controls. Compound **12b** was dosed at 25 mg/kg, BID.
[b] Hepatic microsomes.
[c] Cassette oral dosing at 10 mg/kg.

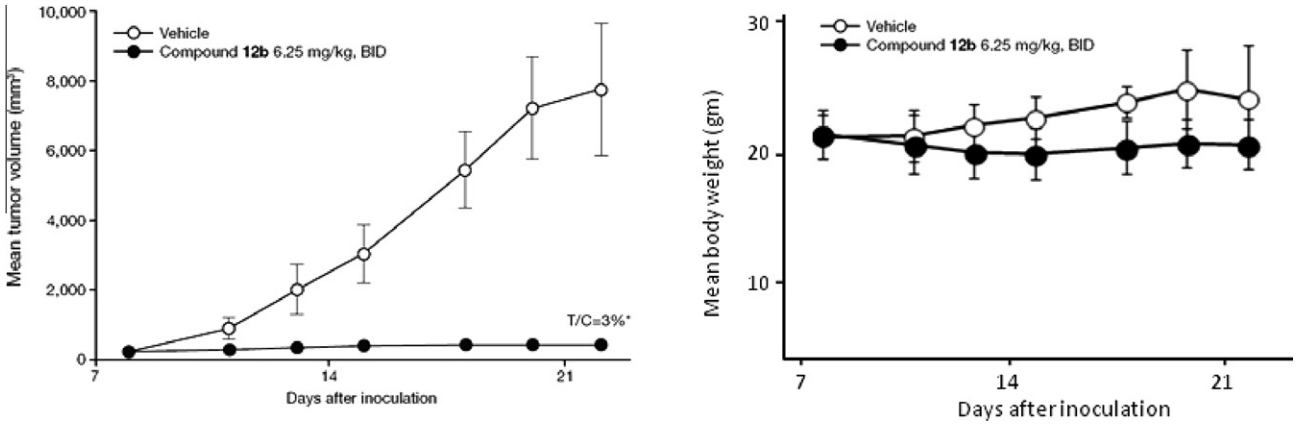


Figure 4. Antitumor activity (left) and body weight change (right) upon treatment with **12b** in nude mice carrying Ptch+/− p53−/− medulloblastoma allograft tumors. Compound **12b** was administrated orally at a dose of 6.25 mg/kg BID for 14 days (●); vehicle controls (○). Each point represents the mean ± SD of duplicate values. *P <0.025 by a 1-tailed Dunnett's test compared to controls.

pharmacokinetic profile. Consistent with the results of in vitro activities and ADME profiles, **12b** strongly suppressed Gli1 mRNA expression after a single cycle of dosing at 25 mg/kg, twice daily (BID).

Compound **12b** demonstrated marked suppression of Gli1 mRNA in the PAN-04 model. This supported in vivo efficacy studies with compound **12b** in a medulloblastoma allograft model generated from Ptch+/− p53−/− mice in which the Hh signaling pathway was constitutively activated. Oral administration of compound **12b** for two weeks at 6.25 mg/kg BID resulted in virtually complete suppression of tumor growth (T/C ratio = 3%; Fig. 4, left) without notable body weight loss (Fig. 4, right). It was suggested that compound **12b** might be effective in the treatment of cancers that are activated by Hh signaling in mice.

4. Conclusions

In these studies, HTS to identify Hh signaling inhibitors resulted in a novel lead compound **9a**, which possesses a thieno[3,2-*c*]quinoline-4-one core structure. Our medicinal chemistry efforts, focusing on maintaining in vitro activity while significantly improving metabolic stability, led to pyrrolo[3,2-*c*]quinoline-4-one **12b** with a characteristic side chain at the 2-position. Compound **12b** showed suppression of Gli1 mRNA expression and virtually complete growth suppression in the medulloblastoma allograft model. This in vivo efficacy encouraged us to seek Hh signaling inhibitors that might be considered as candidates for clinical research in patients with cancers that are characterized by aberrant Hh signaling activity.

5. Experimental section

Melting points were determined on a Büchi melting point apparatus and were not corrected. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Varian Gemini-300 (300 MHz) or Bruker DPX300 (300 MHz) instrument. Chemical shifts are reported as δ values (ppm) downfield from internal tetramethylsilane of the indicated organic solution. Abbreviations for peak multiplicities: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublet; ddd, doublet of doublet of doublets; dt, doublet of triplet; br s, broad singlet; m, multiplet. Coupling constants (*J* values) are given in hertz (Hz). Element analyses were carried out by Takeda Analytical Laboratories, and the results were within 0.4% of theoretical values. LC–MS spectra were obtained on a Shimadzu Corporation LC–MS system (LCMS-2010A). Column chromatography was carried out on silica gel columns (Kieselgel 60, 63–200 mesh, Merck, Darmstadt, Germany) or basic silica gel columns (Chromatorex® NH-DM1020, 100–200 mesh, Fuji Silysia Chemical Ltd., Kasugai, Japan) or Purif-Pack® columns (SI 60 μM or NH 60 μM, Fuji Silysia). Reactions were monitored by thin layer chromatography (TLC) analysis on silica gel 60F_{254} plates (Merck) or NH TLC plates (Fuji Silysia). X-ray structural analyses were determined using MOE version 2010.10; Chemical Computing Group, Inc., Montreal, Quebec, Canada. *C*log*P* values were calculated by Daylight Software *C*log*P*, version 4.82, Daylight Chemical Information Systems, Inc., Aliso Viejo, CA.

5.1. Ethyl 4-hydroxy-2-oxo-1,2-dihydroquinoline-3-carboxylate (2)

A 20% solution of NaOEt in EtOH (274 g) was added to a solution of methyl anthranilate (121 g, 0.800 mol) and diethyl malonate (128 g, 0.800 mol) in EtOH (900 mL), and the mixture was stirred at room temperature for 30 min. The mixture was heated at 140 °C under stirring for 14 h removing EtOH by a Dean-Stark trap. After cooling, the residual solid was washed with Et_2O and dissolved in water. After removal of insoluble solid by filtration, the filtrate was acidified with 5 M HCl, and the precipitated solid was collected by filtration. The solid was washed with water and dried in vacuo to give the title compound (161 g, 86%) as a pale yellow powder. ^{1}H NMR (DMSO-d_6) δ 1.31 (3H, t, *J* = 7.1 Hz), 4.34 (2H, q, *J* = 7.1 Hz), 7.19–7.29 (2H, m), 7.63 (1H, dt, *J* = 1.2, 7.8 Hz), 7.94 (1H, d, *J* = 8.1 Hz), 11.51 (1H, br s), 13.40 (1H, br s).

5.2. Ethyl 2,4-dichloroquinoline-3-carboxylate (3)

A mixture of **2** (75.0 g, 0.320 mol) and $POCl_3$ (200 mL) was stirred at 110 °C for 6 h. After cooling, the reaction mixture was concentrated in vacuo. The residue was dissolved in a small amount of AcOEt and the mixture was poured into ice water followed by extraction with AcOEt. The extract was washed with 1 M NaOH, water, and brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by basic silica gel column chromatography (AcOEt/hexane = 1/5) to give the title compound (68.0 g, 79%) as a white solid. ^{1}H NMR (DMSO-d_6) δ 1.47 (3H, t, *J* = 7.2 Hz), 4.54 (2H, q, *J* = 7.2 Hz), 7.71 (1H, t, *J* = 7.5 Hz), 7.85 (1H, t, *J* = 7.5 Hz), 8.06 (1H, d, *J* = 8.2 Hz), 8.24 (1H, d, *J* = 8.2 Hz).

5.3. Ethyl 4-chloro-2-oxo-1,2-dihydroquinoline-3-carboxylate (4)

A mixture of **3** (68.0 g, 0.250 mol) and AcONa (21.7 g, 0.260 mol) in AcOH (200 mL) was stirred at 120 °C for 20 h. After the reaction mixture was added to water, the precipitated solid was collected and washed with water to give the title compound (58.8 g, 94%) as a white solid. ^{1}H NMR ($CDCl_3$) δ 1.46 (3H, t, *J* = 7.2 Hz), 4.53 (2H, q, *J* = 7.2 Hz), 7.34 (1H, t, *J* = 7.8 Hz), 7.43 (1H, d, *J* = 8.1 Hz), 7.63 (1H, t, *J* = 7.8 Hz), 8.00 (1H, d, *J* = 8.1 Hz), 12.41 (1H, br s).

5.4. Ethyl 4-chloro-2-oxo-1-(2-oxo-2-phenylethyl)-1,2-dihydroquinoline-3-carboxylate (5a)

NaH (60% in oil, 1.70 g, 41.7 mmol) was added to a solution of compound **4** (10.0 g, 39.7 mmol) in DMF (160 mL) under ice-cooling, and the mixture was stirred for 15 min. Phenacyl bromide (8.70 g, 43.7 mmol) was added under ice-cooling, and the resulting mixture was stirred for 1 h. The reaction mixture was added to water, and the mixture was extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by silica gel column chromatography (AcOEt/hexane = 1/2 to 1/1) to give the title compound (10.5 g, 71%) as a pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.42 (3H, t, *J* = 7.2 Hz), 4.47 (2H, q, *J* = 7.2 Hz), 5.80 (2H, s), 7.02 (1H, d, *J* = 8.1 Hz), 7.34 (1H, t, *J* = 7.8 Hz), 7.53–7.60 (3H, m), 7.65–7.70 (1H, m), 8.06–8.13 (3H, m).

5.5. Ethyl 4-chloro-1-(4-methoxybenzyl)-2-oxo-1,2-dihydroquinoline-3-carboxylate (5b)

Similar to the preparation of **5a**, the title compound (10.8 g, 79%) was obtained as a white powder from **4** (10.0 g, 39.8 mmol). ^{1}H NMR (DMSO-d_6) δ 1.34 (3H, t, *J* = 7.1 Hz), 3.70 (3H, s), 4.40 (2H, q, *J* = 7.1 Hz), 5.48 (2H, s), 6.89 (2H, d, *J* = 8.7 Hz), 7.19 (2H, d, *J* = 8.7 Hz), 7.42 (1H, t, *J* = 7.8 Hz), 7.61 (1H, d, *J* = 8.7 Hz), 7.69–7.75 (1H, m), 8.04 (1H, dd, *J* = 7.8, 1.2 Hz).

5.6. Ethyl 3-hydroxy-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydrothieno[3,2-*c*]quinoline-2-carboxylate (6a)

A mixture of ethyl thioglycolate (6.10 g, 50.5 mmol), 20% solution of NaOEt in EtOH (17.2 g, 50.5 mmol), and EtOH (50 mL) was stirred at room temperature for 5 min. Compound **5a** (9.30 g, 25.3 mmol) was added and the mixture was stirred at room temperature for 18 h. The mixture was acidified with 2 M HCl and the resulting mixture was stirred for 30 min. The precipitated solid was collected by filtration, washed with water and Et_2O to give the title compound (10.0 g, 96%) as a white powder. ^{1}H NMR (DMSO-d_6) δ 1.32 (3H, t, *J* = 7.0 Hz), 4.25–4.35 (2H, m), 6.01 (2H, s), 7.40 (1H, t, *J* = 7.2 Hz), 7.55–7.67 (4H, m), 7.77 (1H, t, *J* = 7.5 Hz), 8.00–8.07 (1H, m), 8.07–8.19 (2H, m), 10.57 (1H, br s).

5.7. Ethyl 3-hydroxy-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydrofuro[3,2-*c*]quinoline-2-carboxylate (6b)

Similar to the preparation of **6a**, the title compound (1.10 g, 69%) was obtained as a white powder from **5a** (1.50 g, 4.06 mmol). ^{1}H NMR (DMSO-d_6) δ 1.34 (3H, t, *J* = 7.1 Hz), 4.35 (2H, q, *J* = 7.1 Hz), 5.96 (2H, s), 7.39 (1H, t, *J* = 7.7 Hz), 7.51 (1H, d, *J* = 8.4 Hz), 7.60–7.67 (3H, m), 7.74–7.79 (1H, m), 8.05 (1H, dd, *J* = 7.7, 1.4 Hz), 8.15–8.18 (2H, m), 10.55 (1H, br s).

5.8. Ethyl 3-hydroxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxylate (6c)

A mixture of **5a** (52.0 g, 0.14 mol), sarcosine ethyl ester hydrochloride (25.6 g, 0.16 mol), Et_3N (28.5 g, 0.28 mol), and EtOH (500 mL) was stirred at 85 °C for 12 h. After cooling, the mixture was diluted with water (2200 mL) and extracted with AcOEt. The combined extracts were washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was crystallized with hexane/AcOEt to give the ethyl 4-[(2-ethoxy-2-oxoethyl)(methyl)amino]-

2-oxo-1-(2-oxo-2-phenylethyl)-1,2-dihydroquinoline-3-carboxylate (51.5 g) as pale yellow crystals. A mixture of the compound (51.5 g) obtained above, 20% solution of NaOEt in EtOH (46.6 g, 0.14 mol), and EtOH (1000 mL) was stirred at 50 °C for 18 h. The mixture was diluted with water (500 mL) then acidified with 1 M HCl (140 mL), the resulting mixture was stirred for 30 min. The precipitated solid was collected by filtration, washed with water and EtOH successively, and dried in vacuo to give the title compound (37.5 g, 66%) as a white solid. ^{1}H NMR (DMSO-d_6) δ 1.33 (3H, t, J = 7.2 Hz), 4.30–4.38 (5H, m), 5.93 (2H, s), 7.30–7.40 (2H, m), 7.48–7.53 (1H, m), 7.61–7.66 (2H, m), 7.73–7.79 (1H, m), 8.15–8.18 (2H, m), 8.34–8.37 (1H, m), 9.00 (1H, s). Anal. calcd for $C_{23}H_{20}N_2O_5$: C, 68.31; H, 4.98; N, 6.93. Found: C, 68.41; H, 5.08; N, 7.11.

5.9. Ethyl 3-hydroxy-5-(4-methoxybenzyl)-1-methyl-4-oxo-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxylate (6d)

A mixture of **5b** (16.5 g, 44.47 mmol), sarcosine ethyl ester hydrochloride (10.2 g, 66.5 mmol), Et_3N (27.5 mL, 0.197 mol), and EtOH (170 mL) was stirred at 85 °C for 18 h. A 20% solution of NaOEt in EtOH (75.3 g, 0.221 mol) was added, and the mixture was stirred at 60 °C for 1 h. The mixture was concentrated in vacuo, and the residue was suspended with water (100 mL). The suspension was acidified with 5 M HCl at 0 °C, and the mixture was stirred at room temperature for 1.5 h. The precipitate was collected by filtration, washed with water and EtOH to give the title compound (14.6 g, 81%) as a brown solid. ^{1}H NMR (DMSO-d_6) δ 1.33 (3H, t, J = 7.0 Hz), 3.69 (3H, s), 4.28 (3H, s), 4.34 (2H, q, J = 7.0 Hz), 5.48 (2H, br s), 6.80–6.93 (2H, m), 7.16 (2H, d, J = 8.7 Hz), 7.23–7.35 (1H, m), 7.43–7.52 (2H, m), 8.33 (1H, d, J = 8.1 Hz), 9.10 (1H, s).

5.10. Ethyl 3-methoxy-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydrothieno[3,2-*c*]quinoline-2-carboxylate (7a)

DBU (4.30 g, 18.3 mmol) was added at room temperature to a stirred solution of compound **6a** (7.00 g, 17.1 mmol) in DMF (150 mL). After stirring for 10 min, MeI (4.00 g, 28.0 mmol) was added. The mixture was stirred at room temperature for 4 h, and concentrated in vacuo. The residue was diluted with water, and extracted with AcOEt/THF. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residual solid was collected and washed with AcOEt/Et_2O to give the title compound (5.70 g, 79%) as a pale green powder. ^{1}H NMR (DMSO-d_6) δ 1.34 (3H, t, J = 7.1 Hz), 3.95 (3H, s), 4.34 (2H, q, J = 7.1 Hz), 5.98 (2H, s), 7.37 (1H, t, J = 7.5 Hz), 7.49 (1H, d, J = 8.7 Hz), 7.57–7.67 (3H, m), 7.76 (1H, t, J = 7.5 Hz), 8.06 (1H, d, J = 7.8 Hz), 8.16–8.19 (2H, m).

The following compounds **7b–e** were prepared in a manner similar to that described for **7a**.

5.11. Ethyl 3-methoxy-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydrofuro[3,2-*c*]quinoline-2-carboxylate (7b)

Yield 23%, white powder. ^{1}H NMR (DMSO-d_6) δ 1.35 (3H, t, J = 7.1 Hz), 4.14 (3H, s), 4.37 (2H, q, J = 7.1 Hz), 5.99 (2H, s), 7.39–7.45 (1H, m), 7.55 (1H, d, J = 8.7 Hz), 7.62–7.68 (3H, m), 7.74–7.80 (1H, m), 8.09 (1H, dd, J = 7.8, 1.5 Hz), 8.16–8.18 (2H, m). Anal. calcd for $C_{23}H_{19}NO_6$: C, 68.14; H, 4.72; N, 3.46. Found: C, 67.91; H, 4.78; N, 3.42.

5.12. Ethyl 3-methoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxylate (7c)

Yield 55%, white powder. ^{1}H NMR (DMSO-d_6) δ 1.35 (3H, t, J = 7.1 Hz), 3.89 (3H, s), 4.29–4.37 (5H, m), 5.96 (2H, s), 7.30–7.40 (2H, m), 7.48–7.53 (1H, m), 7.61–7.66 (2H, m), 7.73–7.78 (1H, m), 8.15–8.18 (2H, m), 8.38 (1H, dd, J = 8.3, 1.4 Hz). Anal. calcd for $C_{24}H_{22}N_2O_5$: C, 68.89; H, 5.30; N, 6.69. Found: C, 68.52; H, 5.26; N, 6.61.

5.13. Ethyl 3-ethoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]-quinoline-2-carboxylate (7d)

Yield 100%, white powder. ^{1}H NMR (DMSO-d_6) δ 1.27–1.37 (6H, m), 4.16 (2H, q, J = 6.9 Hz), 4.29–4.36 (5H, m), 5.95 (2H, s), 7.27–7.39 (2H, m), 7.50 (1H, t, J = 8.0 Hz), 7.63 (2H, t, J = 7.3 Hz), 7.76 (1H, t, J = 7.4 Hz), 8.17 (2H, d, J = 7.3 Hz), 8.38 (1H, d, J = 8.1 Hz).

5.14. Ethyl 3-methoxy-5-(4-methoxybenzyl)-1-methyl-4-oxo-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]-quinoline-2-carboxylate (7e)

Yield 61%, pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.35 (3H, t, J = 7.1 Hz), 3.69 (3H, s), 3.94 (3H, s), 4.26 (3H, s), 4.34 (2H, q, J = 7.1 Hz), 5.51 (2H, br s), 6.86 (2H, d, J = 8.7 Hz), 7.15 (2H, d, J = 8.7 Hz), 7.25–7.31 (1H, m), 7.47 (2H, d, J = 3.6 Hz), 8.34 (1H, d, J = 8.1 Hz).

5.15. 3-Methoxy-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydrothieno[3,2-*c*]quinoline-2-carboxylic acid (8a)

A solution of **7a** (5.70 g, 13.5 mmol) and 2 M NaOH (60 mL) in THF (200 mL)/EtOH (100 mL) was stirred at room temperature for 18 h. The reaction mixture was neutralized with 2 M HCl, and the resulting solid was collected by filtration. The solid was washed with water and Et_2O to give the title compound (4.20 g, 79%) as a pale pink powder. ^{1}H NMR (DMSO-d_6) δ 3.93 (3H, s), 5.98 (2H, s), 7.35 (1H, t, J = 7.8 Hz), 7.48 (1H, d, J = 8.7 Hz), 7.56–7.67 (3H, m), 7.77 (1H, t, J = 7.2 Hz), 8.04 (1H, dd, J = 8.1, 1.2 Hz), 8.16–8.19 (2H, m), 13.43 (1H, s).

The following compounds **8b–e** were prepared in a manner similar to that described for **8a**.

5.16. 3-Methoxy-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydrofuro[3,2-*c*]quinoline-2-carboxylic acid (8b)

Yield 72%, white powder. ^{1}H NMR (DMSO-d_6) δ 4.12 (3H, s), 5.99 (2H, s), 7.42 (1H, t, J = 7.6 Hz), 7.54 (1H, d, J = 7.8 Hz), 7.62–7.67 (3H, m), 7.77 (1H, t, J = 7.8 Hz), 8.07 (1H, dd, J = 7.6, 1.5 Hz), 8.16–8.18 (2H, m), 13.35 (1H, br s).

5.17. 3-Methoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxylic acid (8c)

Yield 91%, white powder. ^{1}H NMR (DMSO-d_6) δ 3.88 (3H, s), 4.31 (3H, s), 5.95 (2H, s), 7.29–7.39 (2H, m), 7.46–7.52 (1H, m), 7.61–7.66 (2H, m), 7.73–7.78 (1H, m), 8.16–8.18 (2H, m), 8.38 (1H, dd, J = 8.3, 1.1 Hz), 12.92 (1H, s).

5.18. 3-Ethoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxylic acid (8d)

Yield 89%, white powder. ^{1}H NMR (DMSO-d_6) δ 1.27 (3H, t, J = 7.0 Hz), 4.16 (2H, q, J = 7.0 Hz), 4.31 (3H, s), 5.95 (2H, s), 7.27–7.39 (2H, m), 7.49 (1H, t, J = 7.4 Hz), 7.63 (2H, t, J = 7.4 Hz), 7.76 (1H, t, J = 7.4 Hz), 8.15–8.18 (2H, m), 8.38 (1H, d, J = 7.2 Hz), 12.83 (1H, s). Anal. calcd for $C_{23}H_{20}N_2O_5 \bullet H_2O$: C, 65.39; H, 5.25; N, 6.63. Found: C, 65.65; H, 4.95; N, 6.73.

5.19. 3-Methoxy-5-(4-methoxybenzyl)-1-methyl-4-oxo-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxylic acid (8e)

Yield 96%, pale yellow solid. ^{1}H NMR (DMSO-d_6) δ 3.70 (3H, s), 3.94 (3H, s), 4.28 (3H, s), 5.51 (2H, br s), 6.86 (2H, d, J = 8.7 Hz), 7.15

(2H, d, J = 8.7 Hz), 7.25–7.30 (1H, m), 7.46 (2H, d, J = 3.9 Hz), 8.34 (1H, d, J = 8.4 Hz), 12.90 (1H, br s).

5.20. 3-Methoxy-4-oxo-5-(2-oxo-2-phenylethyl)-*N*-(2-pyrrolidin-1-ylethyl)-4,5-dihydrothieno[3,2-*c*]quinoline-2-carboxamide (9a)

EDC (94 mg, 0.49 mmol) was added to a mixture of **8a** (130 mg, 0.33 mmol), 1-(2-aminoethyl)-pyrrolidine (56 mg, 0.49 mmol), and HOBt (67 mg, 0.49 mmol) in DMF (4 mL), and the mixture was stirred at room temperature for 18 h. The reaction mixture was diluted with saturated $NaHCO_3$ aq and extracted twice with AcOEt. The combined organic extracts were washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by basic silica gel column chromatography (hexane/AcOEt = 1/1 to 0/1), and crystallized from hexane/AcOEt to give the title compound (100 mg, 62%) as white crystals; mp. 190 °C. ^{1}H NMR (DMSO-d_6) δ 1.72 (4H, br s), 2.49 (4H, m), 2.64 (2H, t, J = 6.3 Hz), 3.44 (2H, t, J = 5.9 Hz), 4.01 (3H, s), 6.00 (2H, s), 7.35 (1H, t, J = 7.8 Hz), 7.49 (1H, d, J = 8.1 Hz), 7.55–7.67 (3H, m), 7.76 (1H, t, J = 7.5 Hz), 8.05 (1H, dd, J = 8.1, 1.5 Hz), 8.16–8.19 (3H, m). Anal. calcd for $C_{27}H_{27}N_3O_4S$: C, 66.24; H, 5.56; N, 8.58. Found: C, 66.08; H, 5.50; N, 8.58.

The following compounds **9b-h** were prepared in a manner similar to that described for **9a**.

5.21. 3-Methoxy-4-oxo-5-(2-oxo-2-phenylethyl)-*N*-(2-pyrrolidin-1-ylethyl)-4,5-dihydrofuro[3,2-*c*]quinoline-2-carboxamide (9b)

Yield 66%, white crystals; mp 171 °C (recrystallized from AcOEt). ^{1}H NMR (DMSO-d_6) δ 1.69–1.73 (4H, m), 2.49–2.51 (4H, m), 2.61 (2H, t, J = 6.6 Hz), 3.40–3.46 (2H, m), 4.15 (3H, s), 6.00 (2H, s), 7.43 (1H, t, J = 8.1 Hz), 7.54 (1H, d, J = 8.4 Hz), 7.60–7.67 (3H, m), 7.74–7.79 (1H, m), 8.06 (1H, t, J = 8.6 Hz), 8.15–8.19 (3H, m). LC–MS: m/z = 474 (MH^+). Anal. calcd for $C_{27}H_{27}N_3O_5$: C, 68.48; H, 5.75; N, 8.87. Found: C, 68.32; H, 5.68; N, 8.97.

5.22. 3-Methoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-*N*-(2-pyrrolidin-1-ylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxamide (9c)

Yield 78%, white crystals; mp 204 °C (recrystallized from AcOEt). ^{1}H NMR (DMSO-d_6) δ 1.65–1.75 (4H, m), 2.48–2.55 (4H, m), 2.62 (2H, t, J = 6.2 Hz), 3.39–3.45 (2H, m), 3.97 (3H, s), 4.37 (3H, s), 5.97 (2H, s), 7.29–7.39 (2H, m), 7.45–7.50 (1H, m), 7.61–7.66 (2H, m), 7.73–7.78 (1H, m), 8.16–8.18 (3H, m), 8.39 (1H, dd, J = 8.1, 1.2 Hz). LC–MS: m/z = 487 (MH^+). Anal. calcd for $C_{28}H_{30}N_4O_4$: C, 69.12; H, 6.21; N, 11.51. Found: C, 69.16; H, 6.20; N, 11.59.

5.23. 3-Ethoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-*N*-(2-pyrrolidin-1-ylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxamide (9d)

Yield 18%, white crystals; mp 217 °C (recrystallized from AcOEt/THF). ^{1}H NMR (DMSO-d_6) δ 1.32 (3H, t, J = 7.0 Hz), 1.65–1.75 (4H, m), 2.40–2.55 (4H, m), 2.62 (2H, t, J = 6.0 Hz), 3.05–3.47 (2H, m), 4.29 (2H, q, J = 7.0 Hz), 4.39 (3H, s), 5.96 (2H, s), 7.29–7.39 (2H, m), 7.48 (1H, t, J = 7.8 Hz), 7.63 (2H, t, J = 7.7 Hz), 7.76 (1H, t, J = 7.4 Hz), 8.12–8.19 (3H, m), 8.38 (1H, d, J = 8.4 Hz). LC–MS: m/z = 501 (MH^+). Anal. calcd for $C_{29}H_{32}N_4O_4$: C, 69.58; H, 6.44; N, 11.19. Found: C, 69.83; H, 6.26; N, 10.63.

5.24. 3-Methoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-*N*-(2-piperidin-1-ylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxamide (9e)

Yield 90%, white crystals; mp 230 °C (recrystallized from THF/EtOH). ^{1}H NMR (DMSO-d_6) δ 1.30–1.65 (6H, m), 2.30–2.60 (6H, m), 3.40–3.45 (2H, m), 4.01 (3H, s), 4.38 (3H, s), 5.97 (2H, s), 7.29–7.39 (2H, m), 7.45–7.51 (1H, m), 7.63 (2H, t, J = 7.5 Hz), 7.73–7.78 (1H, m), 8.11–8.19 (3H, m), 8.38 (1H, dd, J = 8.4, 1.2 Hz). LC–MS: m/z = 501 (MH^+). Anal. calcd for $C_{29}H_{32}N_4O_4 \cdot 0.2H_2O$: C, 69.08; H, 6.48; N, 11.11. Found: C, 69.06; H, 6.45; N, 11.09.

5.25. 3-Methoxy-1-methyl-*N*-(2-morpholin-4-ylethyl)-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxamide (9f)

Yield 82%, white crystals; mp 228 °C (recrystallized from AcOEt/THF). ^{1}H NMR (DMSO-d_6) δ 2.40–2.55 (6H, m), 3.40–3.50 (2H, m), 3.61 (4H, t, J = 4.2 Hz), 4.01 (3H, s), 4.37 (3H, s), 5.97 (2H, s), 7.29–7.40 (2H, m), 7.48 (1H, t, J = 7.7 Hz), 7.64 (2H, t, J = 7.4 Hz), 7.76 (1H, t, J = 7.4 Hz), 8.12–8.19 (3H, m), 8.38 (1H, d, J = 8.4 Hz). LC–MS: m/z = 503 (MH^+). Anal. calcd for $C_{28}H_{30}N_4O_5$: C, 66.92; H, 6.02; N, 11.15. Found: C, 67.04; H, 5.93; N, 11.28.

5.26. 3-Methoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-*N*-piperidin-4-yl-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxamide (9g)

Yield 85%, white crystals; mp 206 °C (recrystallized from AcOEt/EtOH). ^{1}H NMR (DMSO-d_6) δ 1.34–1.46 (2H, m), 1.78–1.84 (2H, m), 1.90–2.20 (1H, m), 2.50–2.60 (2H, m), 2.85–3.00 (2H, m), 3.80–3.95 (1H, m), 3.98 (3H, s), 4.32 (3H, s), 5.97 (2H, s), 7.29–7.39 (2H, m), 7.45–7.50 (1H, m), 7.63 (2H, t, J = 7.5 Hz), 7.76 (1H, t, J = 7.5 Hz), 7.86 (1H, d, J = 7.8 Hz), 8.16–8.18 (2H, m), 8.37 (1H, dd, J = 8.4, 1.2 Hz). LC–MS: m/z = 473 (MH^+). Anal. calcd for $C_{27}H_{28}N_4O_4 \cdot 1.0H_2O$: C, 66.11; H, 6.16; N, 11.42. Found: C, 65.82; H, 6.05; N, 11.32.

5.27. *tert*-Butyl 4-({[3-methoxy-5-(4-methoxybenzyl)-1-methyl-4-oxo-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinolin-2-yl]carbonyl}amino)piperidine-1-carboxylate (9h)

Yield 93%, white powder. ^{1}H NMR (DMSO-d_6) δ 1.33–1.58 (11H, m), 1.76–1.94 (2H, m), 2.83–3.09 (2H, m), 3.69 (3H, s), 3.79–3.95 (2H, m), 3.95–4.11 (4H, m), 4.27 (3H, s), 5.52 (2H, br s), 6.81–6.91 (2H, m), 7.14 (2H, d, J = 8.7 Hz), 7.22–7.32 (1H, m), 7.38–7.53 (2H, m), 7.97 (1H, d, J = 7.9 Hz), 8.33 (1H, d, J = 7.9 Hz). LC–MS: m/z = 575 (MH^+).

5.28. 3-Methoxy-5-(4-methoxybenzyl)-1-methyl-4-oxo-*N*-piperidin-4-yl-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxamide hydrochloride (9i)

4 M HCl in AcOEt solution (5 mL) was added to a solution of **9h** (500 mg, 0.87 mmol) in AcOEt (15 mL), and the mixture was stirred at room temperature for 4 h. The precipitate was collected by filtration, washed with diethyl ether, and dried in vacuo to give the title compound (440 mg, 99%) as a pale pink powder. ^{1}H NMR (DMSO-d_6) δ 1.78 (2H, q, J = 10.3 Hz), 2.05 (2H, d, J = 10.8 Hz), 3.06 (2H, t, J = 10.7 Hz), 3.26–3.33 (2H, m), 3.63 (3H, s), 4.02 (3H, s), 4.09–4.13 (1H, m), 4.24 (3H, s), 5.40–5.60 (2H, m), 6.86 (2H, d, J = 8.7 Hz), 7.14 (2H, d, J = 8.7 Hz), 7.25–7.30 (1H, m), 7.42–7.49 (2H, m), 8.15 (1H, d, J = 7.5 Hz), 8.33 (1H, d, J = 7.8 Hz), 8.60–8.90 (2H, m). LC–MS: m/z = 475 (MH^+-HCl).

5.29. *N*-[1-(2-Hydroxyethyl)piperidin-4-yl]-3-methoxy-5-(4-methoxybenzyl)-1-methyl-4-oxo-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxamide (10)

A mixture of **9i** (9.39 g, 18.4 mmol), 2-bromoethanol (3.25 mL, 45.6 mmol) and K_2CO_3 (10.2 g, 73.6 mmol) in DMF (250 mL) was stirred at 80 °C for 16 h. After cooling, the reaction mixture was concentrated in vacuo and the residue was partitioned between saturated $NaHCO_3$ aq and AcOEt. The aqueous layer was separated and extracted with AcOEt/THF. The combined extracts were washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residual precipitate was collected by filtration, and washed with hexane/AcOEt to give the title compound (7.75 g, 81%) as white powder. ^{1}H NMR (DMSO-d_6) δ 1.43–1.71 (2H, m), 1.77–1.94 (2H, m), 2.08–2.26 (2H, m), 2.40 (2H, t, J = 6.3 Hz), 2.70–2.86 (2H, m), 3.50 (2H, q, J = 6.2 Hz), 3.69 (3H, s), 3.73–3.94 (1H, m), 4.04 (3H, s), 4.29 (3H, s), 4.37 (1H, t, J = 5.4 Hz), 5.52 (2H, br s), 6.78–6.91 (2H, m), 7.14 (2H, d, J = 8.7 Hz), 7.22–7.33 (1H, m), 7.38–7.53 (2H, m), 7.90 (1H, d, J = 7.7 Hz), 8.33 (1H, d, J = 7.9 Hz). LC–MS: m/z = 519 (MH^+).

5.30. 2-(4-{[(3-Methoxy-1-methyl-4-oxo-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinolin-2-yl)carbonyl]-amino}piperidin-1-yl)ethyl acetate (11)

A mixture of **10** (498 mg, 0.959 mmol), anisole (0.4 mL, 3.68 mmol), TfOH (1 mL), and TFA (4 mL) was stirred at 80 °C for 1 h. After the mixture was concentrated in vacuo, the residue was quenched with aqueous $NaHCO_3$ solution and diluted with AcOEt/THF = 1/1 solution (40 mL). The organic layer was separated and the aqueous layer was extracted with AcOEt/THF = 1/1 solution. NaCl (15 g) was added to the aqueous layer and was extracted with THF. The combined organic layer was dried over $MgSO_4$, and concentrated in vacuo. Et_3N (0.4 mL, 2.87 mmol) and acetyl chloride (164 μL, 2.31 mmol) were added at 0 °C to a suspension of the residue in THF (10 mL). The mixture was stirred at 0 °C for 2 h, Et_3N (0.4 mL, 2.87 mmol) and acetyl chloride (164 μL, 2.31 mmol) were further added. After stirring at 0 °C for 2 h, the mixture was quenched with aqueous $NaHCO_3$ solution (30 mL) and extracted with AcOEt. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/AcOEt = 1/2 to AcOEt/MeOH = 4/1) to give the title compound (221 mg, 52%) as a pale yellow powder. ^{1}H NMR (DMSO-d_6) δ 1.46–1.66 (2H, m), 1.76–1.92 (2H, m), 2.02 (3H, s), 2.13–2.30 (2H, m), 2.55 (2H, t, J = 5.9 Hz), 2.69–2.86 (2H, m), 3.70–3.92 (1H, m), 4.03 (3H, s), 4.11 (2H, t, J = 5.9 Hz), 4.29 (3H, s), 7.15–7.28 (1H, m), 7.35–7.52 (2H, m), 7.85 (1H, d, J = 7.7 Hz), 8.24 (1H, d, J = 8.1 Hz), 11.33 (1H, s). LC–MS: m/z = 441 (MH^+).

5.31. *N*-[1-(2-Hydroxyethyl)piperidin-4-yl]-3-methoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxamide (12a)

A mixture of **9g** (200 mg, 0.399 mmol), 2-bromoethanol (42.5 μL, 0.599 mmol), K_2CO_3 (248 mg, 1.80 mmol), and DMF (5 mL) was stirred at 100 °C for 15 h. The reaction mixture was diluted with water, and extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by basic silica gel column chromatography (AcOEt) followed by crystallization from AcOEt/EtOH to give the title compound (68.0 mg, 33%) as white crystals; mp 201 °C. ^{1}H NMR (DMSO-d_6) δ 1.51–1.62 (2H, m), 1.75–1.90 (2H, m), 2.18 (2H, t, J = 10.5 Hz), 2.40 (2H, t, J = 5.9 Hz), 2.70–2.85 (2H, m), 3.47–3.52 (2H, m), 3.75–3.90 (1H, m), 3.98 (3H, s), 4.32–4.38 (4H, m), 5.97 (2H, s), 7.29–7.39 (2H, m), 7.47 (1H, t, J = 7.6 Hz), 7.63 (2H, t, J = 7.6 Hz), 7.76 (1H, t, J = 7.1 Hz), 7.87 (1H, d, J = 7.5 Hz), 8.17 (2H, d, J = 7.6 Hz), 8.37 (1H, d, J = 8.4 Hz). LC–MS: m/z = 517 (MH^+). Anal. calcd for $C_{29}H_{32}N_4O_5$: C, 67.43; H, 6.24; N, 10.85. Found: C, 67.28; H, 6.20; N, 10.79.

5.32. *N*-[1-(Hydroxyacetyl)piperidin-4-yl]-3-methoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxamide (12b)

Acetoxyacetyl chloride (47.3 μL, 0.440 mmol) was added to a mixture of **9g** (200 mg, 0.423 mmol), Et_3N (122 μL, 0.880 mmol), and THF (10 mL) at 0 °C and the mixture was stirred at room temperature for 15 h. The reaction mixture was diluted with AcOEt, washed with water and brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by basic silica gel column chromatography (AcOEt) followed by crystallization from AcOEt/THF to give 2-[4-({[3-methoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinolin-2-yl]carbonyl}amino)piperidin-1-yl]-2-oxoethyl acetate (171 mg, 75%) as a white powder. The compound obtained was dissolved in 8 M NaOH aq (1 mL), THF (2 mL), and EtOH (7 mL). After stirring at room temperature for 2 h, the reaction mixture was diluted with water, acidified with 6 M HCl aq, and extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by basic silica gel column chromatography (AcOEt) followed by crystallization from AcOEt/THF to give the title compound (59.2 mg, 51%) as white crystals; mp 223 °C. ^{1}H NMR (DMSO-d_6) δ 1.35–1.65 (2H, m), 1.80–2.00 (2H, m), 2.85–3.00 (1H, m), 3.05–3.25 (1H, m), 3.60–3.75 (1H, m), 3.97 (3H, s), 4.00–4.30 (4H, m), 4.31 (3H, s), 4.51 (1H, t, J = 5.3 Hz), 5.97 (2H, s), 7.29–7.39 (2H, m), 7.48 (1H, t, J = 7.6 Hz), 7.63 (2H, t, J = 7.6 Hz), 7.76 (1H, t, J = 7.4 Hz), 7.95 (1H, d, J = 8.4 Hz), 8.17 (2H, d, J = 7.6 Hz), 8.37 (1H, d, J = 8.1 Hz). LC–MS: m/z = 531 (MH^+). Anal. calcd for $C_{29}H_{30}N_4O_6$: C, 65.65; H, 5.70; N, 10.56. Found: C, 65.60; H, 5.62; N, 10.46.

5.33. *N*-[1-(2-hydroxyethyl)piperidin-4-yl]-3-methoxy-1-methyl-4-oxo-5-(2-oxobutyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxamide (12c)

tert-BuONa (89.3 mg, 0.929 mmol) was added to a suspension of **11** (299 mg, 0.679 mmol) in DME (2.4 mL)/DMF (0.6 mL) and the mixture was stirred at 0 °C for 30 min. LiBr (142 mg, 1.64 mmol) was added, and the mixture was stirred at room temperature for 10 min. 1-Bromo-2-butanone (76.6 μL, 0.750 mmol) was added and the mixture was stirred at room temperature for 65 h. After addition of 1 M NaOH aq (1.5 mL) and MeOH (1 mL), the mixture was stirred at room temperature for 30 min, and concentrated in vacuo. The residue was partitioned between AcOEt/THF = 3/1 solution (20 mL) and aq $NaHCO_3$ solution (20 mL). The organic layer was separated, and the aq layer was extracted with AcOEt/THF = 3/1 solution (20 mL × 3). The organic layer was combined, washed with brine (10 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was suspended with AcOEt (10 mL) and DMF (1 mL) at 60 °C, and insoluble material was removed by filtration. The filtrate was concentrated in vacuo, and purified by basic silica gel column chromatography (hexane/AcOEt = 1/2 to AcOEt/MeOH = 9/1). Crystallization from IPE/AcOEt gave the title compound (48.7 mg, 15%) as white crystals; mp 191 °C. ^{1}H NMR (DMSO-d_6) δ 1.01 (3H, t, J = 7.3 Hz), 1.45–1.67 (2H, m), 1.75–1.91 (2H, m), 2.08–2.25 (2H, m), 2.39 (2H, t, J = 6.3 Hz), 2.70 (2H, q, J = 7.2 Hz), 2.70–2.85 (2H, m), 3.42–3.57 (2H, m), 3.71–3.91 (1H, m), 3.98 (3H, s), 4.29 (3H, s), 4.38 (1H, t, J = 5.3 Hz), 5.29 (2H, s), 7.24–7.37 (2H, m), 7.43–7.56 (1H, m), 7.87 (1H, d, J = 7.9 Hz), 8.28–8.40 (1H, m). LC–MS: m/z = 469 (MH^+). Anal. calcd for $C_{25}H_{32}N_4O_5$: C, 64.09; H, 6.88; N, 11.96. Found: C, 63.73; H, 6.83; N, 11.81.

5.34. *N*-[1-(2-Hydroxyethyl)piperidin-4-yl]-3-methoxy-1-methyl-4-oxo-5-(2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxamide (12d)

In a similar manner to the preparation of **12c**, the title compound (8.2 mg, 2.4%) was obtained as beige crystals from **11** (299 mg, 0.678 mmol); mp 163 °C (crystallized from IPE/AcOEt). ^{1}H NMR (DMSO-d_6) δ 1.46–1.67 (2H, m), 1.76–1.91 (2H, m), 2.09–2.25 (2H, m), 2.40 (2H, t, J = 6.3 Hz), 2.67–2.84 (2H, m), 2.86–2.99 (2H, m), 3.44–3.55 (2H, m), 3.70–3.91 (1H, m), 3.98 (3H, s), 4.28 (3H, s), 4.37 (1H, t, J = 5.4 Hz), 4.45–4.58 (2H, m), 7.17–7.28 (1H, m), 7.28–7.40 (5H, m), 7.56–7.67 (1H, m), 7.69–7.78 (1H, m), 7.86 (1H, d, J = 7.7 Hz), 8.36 (1H, dd, J = 8.4, 1.2 Hz). LC–MS: m/z = 503 (MH^+). Anal. calcd for $C_{29}H_{34}N_4O_4{\cdot}0.5H_2O$: C, 68.08; H, 6.90; N, 10.95. Found: C, 67.77; H, 6.76; N, 10.72.

5.35. Gli-luc reporter assay

In vitro activities of these compounds were evaluated using a luciferase reporter in NIH3T3 cells carrying a stably transfected Gli-reporter construct (Gli-luc reporter cell line).[16] IH3T3/Gli-luc cells were maintained in DMEM containing 10% FBS, 500 μg/mL G418, and 0.1% gentamicin solution (Invitrogen Corp., Carlsbad, CA, USA). Cells were seeded onto collagen-coated 384-well plates at 7.5×10^3cells/well and cultured overnight in 25 μL of DMEM containing 10% FBS under 5% CO_2 at 37 °C. After incubation, 20 μL of recombinant mouse Shh-N (2.5 μg/mL in DMEM containing 2% FBS) and 5 μL of serially diluted compounds (10× solution; 0.0003–10 μM in DMEM) were added to each well to achieve the final concentrations of 5.8% FBS, 1 μg/mL of Shh-N, and 0.03–1000 nM of the test compounds (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 1 μg/mL Shh-N to serve as 0% or 100% inhibition controls, respectively (n = 10 wells). Luciferase activities of reporter cells were measured by Bright-Glo™ (Promega Corp [Madison, WI, USA]) using the EnVision® plate reader (PerkinElmer, Inc [Waltham, MA, USA]).

5.36. Smo binding assay

293T cells were transfected with pCMV-HA/hSmo using Lipofectamine reagent (Lipofectamine™ 2000, Invitrogen). In brief, 45 μL of Lipofectamine 2000 and 22.5 μg of pCMV-HA/hSmo were each incubated in 1.5 mL of Opti-MEM I media (Invitrogen) at room temperature for 5 min and were mixed together. The mixture was incubated for 20 min at room temperature after which it was added to 6×10^6 293T cells in 15 mL of culture medium in a 75 cm^2 cell culture flask. After overnight incubation under 5% CO_2 at 37 °C, cells were detached using Versene (Invitrogen) and resuspended in culture medium. The suspended cells were added to a 96-well plate at 6×10^4 cells/well. After removing the media from each well, 25 μL of a 2× stock solution of the compounds and 25 μL of BODIPY-cyclopamine (4 nM in DMEM containing 1% FBS) were added to the culture which was incubated for 1 h (n = 3 wells per concentration). Final concentrations were 0.03–1000 nM for the compounds and 2 nM for BODIPY-cyclopamine. To define the assay window, 25 μL of media or 25 μL of 2× cyclopamine solution (final concentration 1 μM) was added to the culture in addition to 25 μL of BODIPY cyclopamine to serve as 0% or 100% inhibition controls (n = 5 wells), respectively. After incubation, cells were detached using Versene and were resuspended in PBS supplemented with 2% FBS. Cyclopamine binding competition with the compounds was determined by measuring the intensity of cell fluorescence using the Guava easyCyte System (Millipore Corp, Billerica, MA, USA).

5.37. In vivo pharmacodynamic assay

The in vivo pharmacodynamic assay was conducted in nude mice bearing human primary pancreatic tumors (PAN-04). The tumor line was established by Central Institute for Experimental Animals and test compounds were orally administered twice daily. After 24 h from the first administration, tumors were excised and treated with RNAlater (Ambion). Total RNA samples were isolated using RNeasy Mini kit (Qiagen), and first strand cDNA samples were prepared using a high capacity cDNA transcription kit (Applied Biosystems). The primer sets of qPCR for quantification of stromal Gli1 mRNA (Applied Biosystems) were as follows: Mm00494645_m1 (mouse Gli1), 4352339E (mouse GAPDH).

5.38. In vivo anti-tumor test

Anti-tumor effects of test compounds were evaluated using a mouse medulloblastoma allogeneic transplantation model.[17] The medulloblastoma spontaneously occurred in the cerebellum of 7 to 9-week-old Ptch1 (+/−) and p53 (−/−) double-mutant mice. Ptch1 gene mutant mice (Ptch1tm1Mps/J) were purchased from The Jackson Laboratory, Bar Harbor, ME; and p53 gene mutant mice (P53N4-M; B6.129-Trp53tm/BrdN4) were purchased from Taconic, Hudson, NY. Medulloblastoma allograft tumors were transplanted subcutaneously into nude mice (CAnN.Cg-Foxn1/nu/CrlCrlj; Charles River Laboratories, Yokohama, Japan), and were used for compound testing after several passages in vivo. When tumors reached an average size of 150–250 mm^3, animals were orally dosed with test compounds (0.5% methylcellulose suspension) or vehicle alone twice daily for 2 weeks. Tumor size was measured with an electronic vernier caliper, and tumor volume was calculated based on the longest (a) and shortest (b) tumor dimensions using the formula volume (V) = ($a \times b^2$)/2. The tumor growth rate (T/C%) was calculated as the mean values for [Treated ($V_{end}-V_{start}$)/Control ($V_{end}-V_{start}$)] × 100.

5.39. In vitro metabolism with hepatic microsomes

Metabolic stability was evaluated both in mouse and human microsomes and used according to the manufacturer's instructions. Liver microsomes were purchased from Xenotech, LLC, Lenexa, KS, USA. An incubation mixture consisted of microsomes in 50 mmol/L of KH_2PO_4/K_2HPO_4 phosphate buffer (pH 7.4) and 1 μmol/L of test compound. The concentration of microsomes was 0.2 mg protein/mL. An NADPH-generating system containing 25 mmol/L $MgCl_2$, 25 mmol/L glucose-6-phosphate, 2.5 mmol/L beta-NADP+, and 7.5 unit/mL glucose-6-phosphate dehydrogenase was added to the incubation mixture with a 20% volume of the reaction mixture to initiate the enzyme reaction. After the addition of the NADPH-generating system, the mixture was incubated at 37 °C. The reaction was terminated by the addition of MeCN equivalent to the volume of the reaction mixture. Test compounds in the reaction mixtures were quantified by HPLC equipped with a UV detector or LC–MS /MS. To determine metabolic stabilities, chromatograms were analyzed for parent compound disappearance from the reaction mixtures.

5.40. Pharmacokinetic studies

Test compounds were administered at a dose of 10 mg/kg (in 0.5% methylcellulose) as cassette dosing to non-fasted mice. After oral administration, blood samples were collected and centrifuged to obtain the plasma fraction. Plasma samples were deproteinized with MeCN containing an internal standard. After centrifugation, the supernatant was diluted with a mixture of 0.01 mol/L $HCOONH_4$ solution and MeCN (9:1, v/v) and centrifuged again.

The compound concentrations in the supernatant were measured by LC–MS/MS.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.07.039.

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