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Discovery of the investigational drug TAK-441, a pyrrolo[3,2-*c*]pyridine derivative, as a highly potent and orally active hedgehog signaling inhibitor: Modification of the core skeleton for improved solubility

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ABSTRACT

We recently reported the discovery of the novel pyrrolo[3,2-*c*]quinoline-4-one derivative **1** as a potent inhibitor of Hedgehog (Hh) pathway signaling. However, the PK evaluation of **1** at high dosage (100 mg/kg) revealed the C_{max} value 3.63 μg/mL, likely due to poor solubility of this compound. Efforts to improve solubility by reducing the aromatic ring count of the core system led to *N*-methylpyrrolo[3,2-*c*]pyridine derivative **11**. Further optimization of the 3-alkoxy group led to compound **11d** with acceptable solubility and potent Hh inhibitory activity. Compound **11d** suppressed transcription factor Gli1 *m*RNA expression in tumor-associated stromal tissue and inhibited tumor growth (treatment/control ratio, 3%) in a mouse medulloblastoma allograft model owing to the improved PK profile based on increased solubility. Compound **11d** (TAK-441) is currently in clinical trials for the treatment of advanced solid tumors.

1. Introduction

We reported pyrrolo[3,2-*c*]quinoline-4-one derivative **1** (Table 1) as a potent Hh signaling inhibitor.[1] The phenacyl group at the 5-position is important for its potent in vitro activity, and the *N*-[1-(hydroxyacetyl)piperidinyl] amide side chain at the 2-position provided both high metabolic stability and reduction of human ether-a-go-go related gene (hERG) inhibition, which is important in cardiac repolarization, based on its low basicity.

Compound **1** exhibited potent suppression of tumor growth in in vivo experiments in a mouse medulloblastoma allograft model. However, the pharmacokinetic (PK) profile of **1** at high dose in mice (100 mg/kg) showed poor oral absorption that was not proportional to dose (AUC_{0-24h} 32.3 μgh/mL; Table 1). Considering that this poor oral absorption could be attributed to low solubility (8.4 μg/mL at pH 6.8), we sought to improve the solubility while maintaining Hh pathway inhibitory activity as shown in Figure 1. In general, compounds with a tricyclic core show poor solubility and often low bioavailability.[2] Thus, we removed the benzene ring on pyrrolo[2,3-*c*]quinoline-4-one in order to decrease planarity. In addition, we speculated that the residual substituent at the 3-position was available for modification of the physicochemical properties. Our prior investigations demonstrated that substituents at the 2- and 5-positions were fixed because they played important roles in potency and metabolic stability as mentioned above. Herein, we describe the design and synthesis of a novel series of pyrrolo[3,2-*c*]pyridine derivatives leading to the identification of our clinical candidate, **11d** (TAK-441).

2. Chemistry

We considered that the removal of the benzene ring from compound **1** would improve solubility by reducing planarity. Retrosynthetic analysis (Fig. 2) demonstrated that ketoesters bearing R^1 and R^2 substituents would allow investigation of replacements for the phenyl ring that was being removed.

The general synthesis of the compounds in this study is outlined below. Pyridone **4**, except for commercially available **4c**, was prepared from **2**. Ketoester **2** was converted to **3** by amination. Cyclization of **3** with diethyl malonate afforded **4** in 60–92% yield. Monochlorination of **4** was achieved using phosphoryl chloride in 17–60% yields. Alkylation of **5** with phenacyl bromide and potassium carbonate proceeded in low yield (7–23%) because of the production of O-phenacylated compound as a major product. Substitution and cyclization of **6** was conducted with sarcosine ethyl ester hydrochloride under basic conditions[3] to obtain pyrrolo[3,2-*c*]pyridine **7**. The hydroxyl group at the 3-position of **7** was

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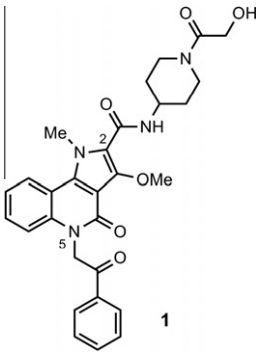
Table 1
Activity profile and solubility of compound **1**



Gli-luc reporter IC_{50}[a] (nM)	In vivo PD Gli1 *m*RNA[b] (% of ctrl)	Solubility[c] (μg/mL)	Mouse PK 10 mg/kg[d]		Mouse PK 100 mg/kg[e]	
			C_{max}	AUC (μgh/mL)	C_{max}	AUC (μgh/mL)
4.6	5	8.4	2.65	12.1	3.63	32.3

[a] IC_{50} values are the mean of four measurements.
[b] The value is the Gli1 *m*RNA expression level at 25 mg/kg BID of compound **1** compared to controls.
[c] Measured at pH 6.8 (Japanese Pharmacopoeia second fluid).
[d] Cassette dosing, AUC_{0-8h}.
[e] BALB/c-nu/nu mice, AUC_{0-24h}.

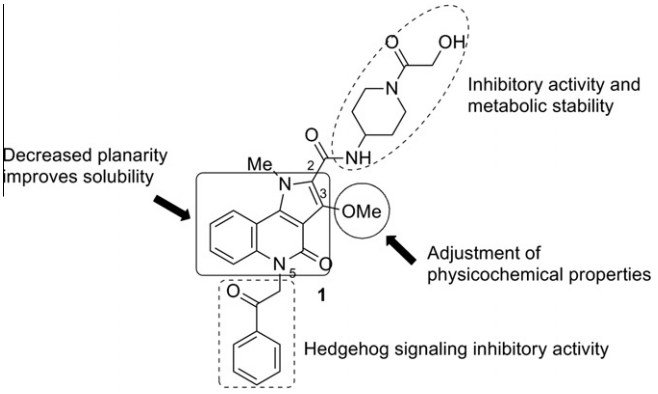


Figure 1. Compound design strategy for improving solubility and maintaining potency.

treated with various alkylating reagents to afford corresponding alkoxy derivatives **8a**–**e**, **8i**, and **8j**. Ethyl derivative **8h** was prepared by treatment of **7a** with trifluoromethanesulfonic anhydride and afforded triflate **8f**. Following Stille coupling of the obtained triflate **8f** with vinyltributyltin, ethenyl derivative **8g** was treated with palladium on carbon under hydrogen atmosphere to give the 3-ethyl derivative **8h**. Finally, saponification of **8** afforded the corresponding carboxylic acid **9** in 23–99% yield (Scheme 1).

Amidation of carboxylic acid **9** is shown in Scheme 2. Compounds **11e** and **11j** were synthesized from corresponding carboxylic acids **9e** and **9j** by stepwise acylation. Compounds **11a**–**d**, **11h**, and **11i** were obtained by condensation of corresponding carboxylic acid **9** with amine **13** directly. The requisite **13** was prepared from 4-Boc-aminopiperidine (**12**) in three steps: acylation with acetoxyacetyl chloride, followed by removal of the acetyl and *tert*-butoxycarbonyl groups in 80% yield.

3. Results and discussion

In vitro activities of compounds **11a**–**j** were evaluated using luciferase reporter activities in NIH3T3 cells carrying a stably-transfected Gli-reporter construct, designated the Gli-luc reporter cell line. First, modification of the benzene ring to cyclohexane in the core structure was examined. In the Gli-luc reporter assay, cyclohexene **11i** showed an approximately 20-fold decreased activity compared with that of **1** (Table 2). This result implied that a substituent with specific size and shape would be required for maintaining the potent Gli-luc reporter activity. Downsizing the cyclohexene ring to a monomethyl group (R^1) was effective in maintaining activity (**11j** vs **11i**). In addition, solubility of **11j**

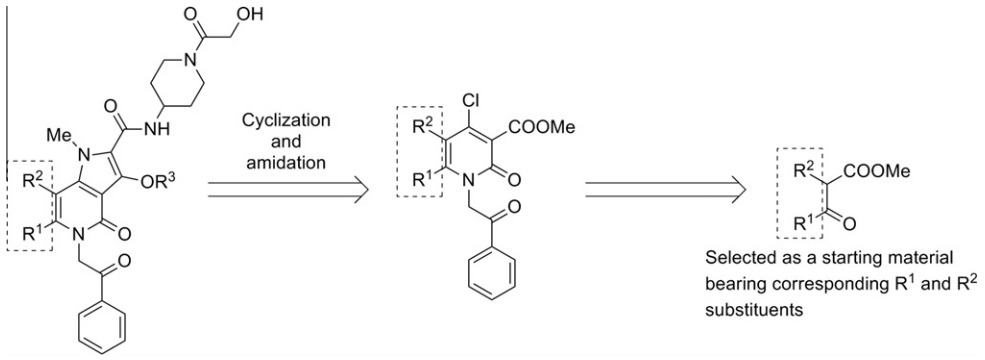
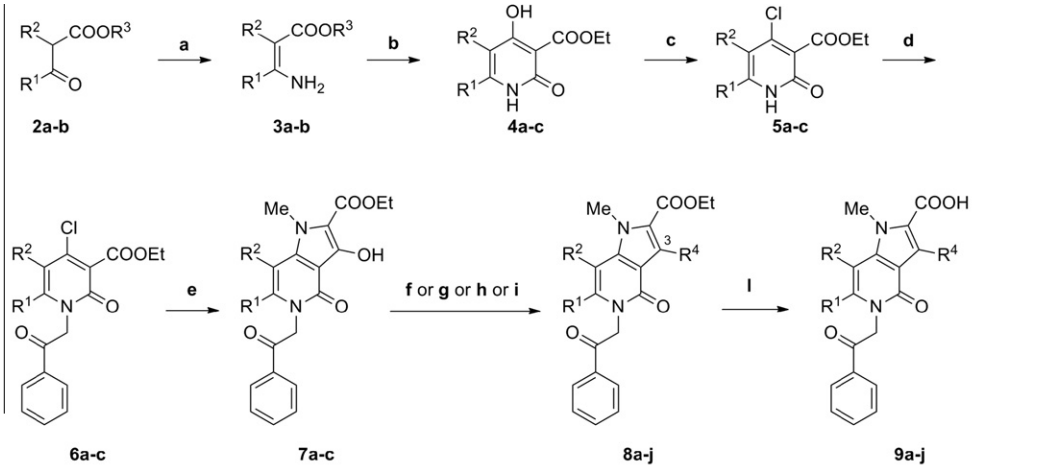


Figure 2. Synthetic plan.



cmpds	R^1	R^2	R^3	yield
2a 3a	Et	H	Me	92
2b 3b	-$(CH_2)_4$-		Et	60

cmpds	R^1	R^2	yield			
			3 to **4**	**4** to **5**	**5** to **6**	**6** to **7**
4-7a	Et	H	46	60	7	74
4-7b	-$(CH_2)_4$-		58	17	16	-
4-7c	Me	H		44	23	68

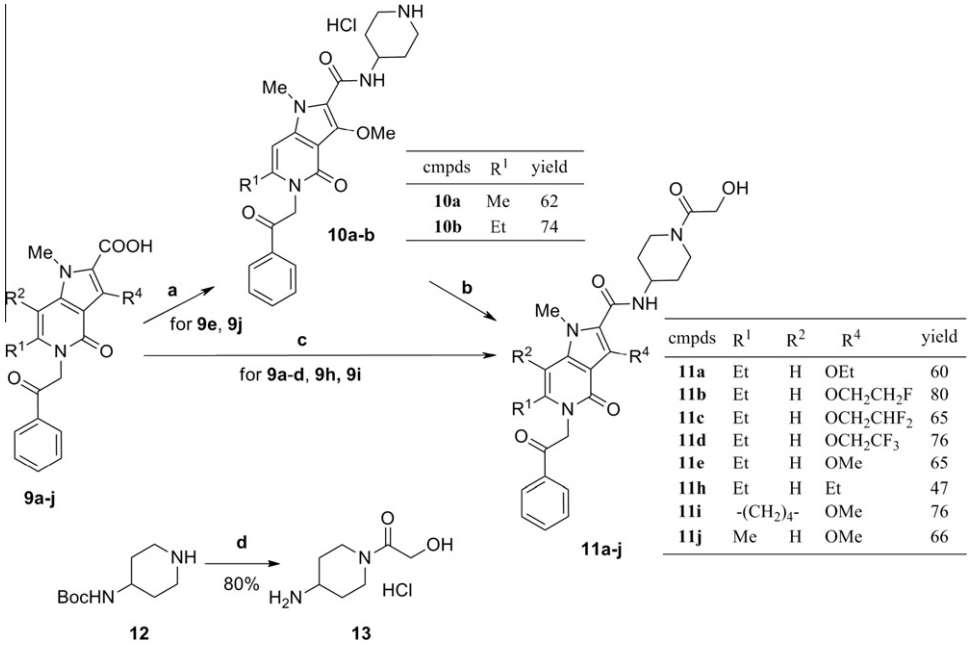
cmpds	R^1	R^2	R^4	yield
8a	Et	H	OEt	82
8b	Et	H	OCH_2CH_2F	77
8c	Et	H	OCH_2CHF_2	77
8d	Et	H	OCH_2CF_3	86
8e	Et	H	OMe	23
8f	Et	H	OTf	59
8g	Et	H	ethenyl	72[1)]
8h	Et	H	Et	100[2)]
8i	-$(CH_2)_4$-		OMe	11[3)]
8j	Me	H	OMe	26

j, k

1) yield from **8f**
2) yield from **8g**
3) 2 steps yield from **6b**

cmpds	R^1	R^2	R^4	yield
9a	Et	H	OEt	89
9b	Et	H	OCH_2CH_2F	56
9c	Et	H	OCH_2CHF_2	83
9d	Et	H	OCH_2CF_3	93
9e	Et	H	OMe	79
9h	Et	H	Et	99
9i	-$(CH_2)_4$-		OMe	23
9j	Me	H	OMe	89

Scheme 1. Reagents and conditions: (a) NH_4OAc, MeOH, rt; (b) diethyl malonate, NaOEt, EtOH, xylene, 120 °C, then 150 °C; (c) $POCl_3$ for **4a,b** or $POCl_3$, $BnEt_3NBr$, MeCN, 40 °C, then reflux for **4c**; (d) $PhCOCH_2Br$, K_2CO_3, DMF, rt for **5a,c** or $PhCOCH_2Br$, NaH, DMA, rt for **5b**; (e) $MeNHCH_2COOEt{\cdot}HCl$, Et_3N, EtOH, reflux; (f) R′X, DBU, DMF, rt for **8b** (R′ = CH_2CH_2F), **8e, j** (R′ = Me); (g) R'_2SO_4, K_2CO_3, acetone, reflux for **8a** (R′ = Et), **8i** (R′ = Me); (h) R′OTf, Cs_2CO_3, DMF, rt for **8c** (R′ = CH_2CHF_2), **8d** (R′ = CH_2CF_3); (i) Tf_2O, pyridine, 60 °C for **8f**; (j) vinyltributyltin, $Pd(PPh_3)_4$, DMF, 100 °C; (k) Pd/C, H_2, THF/MeOH, rt; (l) NaOH, EtOH, H_2O, 60 °C.



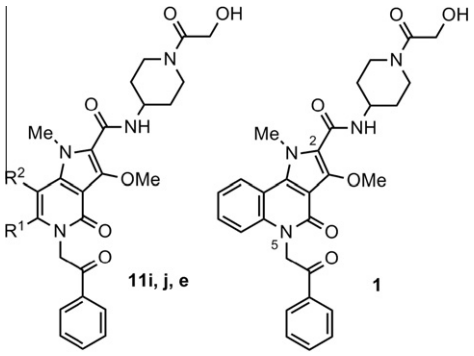
cmpds	R^1	yield
10a	Me	62
10b	Et	74

cmpds	R^1	R^2	R^4	yield
11a	Et	H	OEt	60
11b	Et	H	OCH_2CH_2F	80
11c	Et	H	OCH_2CHF_2	65
11d	Et	H	OCH_2CF_3	76
11e	Et	H	OMe	65
11h	Et	H	Et	47
11i	-$(CH_2)_4$-		OMe	76
11j	Me	H	OMe	66

Scheme 2. Reagents and conditions: (a) (1) 4-amino-1-Boc-piperidine, EDC, HOBt, DMF, rt, (2) 4 M HCl in AcOEt, AcOEt, rt; (b) (1) $ClCOCH_2OAc$, Et_3N, THF, rt, (2) NaOH, EtOH, H_2O, rt for **11e**, **11j**; (c) **13**, EDC, HOBt, DMF, rt for **11a–d**, **11h**, **11i**; (d) (1) $ClCOCH_2OAc$, Et_3N, THF, 0 °C; (2) 8 M NaOH, EtOH, H_2O, rt; (3) 4 M HCl in AcOEt, AcOEt, rt.

was improved compared with that of **1**, as expected. Introduction of an ethyl group at the 6-position resulted in further enhancement of Hh inhibitory activity without loss of solubility (**11e** vs **11j**). Among the compounds listed in Table 2, we selected for further investigation the 6-ethyl derivative **11e** which had potent activity in the Gli-luc reporter assay and good solubility.

Table 2
Modification of the core ring



Compds	R^1	R^2	Gli-luc reporter IC_{50}[a] (nM)	Solubility[b] (μg/mL)
11i	$-(CH_2)_4-$		96	80
11j	Me	H	14	77
11e	Et	H	5.7	63
1			4.6	8.4

[a] IC_{50} values are the mean of four measurements.
[b] Measured at pH 6.8 (Japanese Pharmacopoeia second fluid).

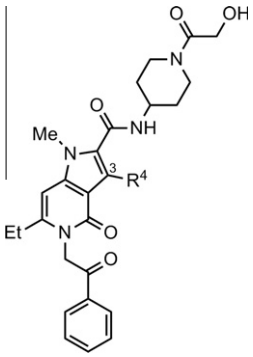
Compound **11e** was examined in an in vivo PD study at 25 mg/kg, BID, but efficacy was relatively poor. We surmised that the solubility of compound **11e** was insufficient to show good efficacy at the higher dose. To further improve solubility, substitutions were made at the 3-position (Table 3). Exchange of the methoxy group in **11e** to an ethyl group significantly decreased activity (**11h** vs **11e**) suggesting that the oxygen atom in the 3-methoxy group in **11e** plays an important role in activity. In general, fluorination of alkyl substituents is known to impact the physicochemical profile by changing lipophilic and electronic factors.[4] Therefore, we investigated the effect of fluorination on solubility and potency using the 3-ethoxy derivative **11a** as a standard compound with good activity and solubility.

Both 2-fluoroethoxy (**11b**) and 2,2-difluoroethoxy (**11c**) groups effectively increased solubility, but exposure (AUC) of these compounds in mice following oral dosing was decreased compared with that of **11a**. On the other hand, trifluoroethoxy derivative **11d** exhibited favorable exposure compared to those of **11a** and **11e**. Furthermore, the oral absorption of **11d** was better than that of **1**, suggesting that the improvement of solubility contributed to the improved PK profile at the higher dose (Table 4). Thus, we selected **11d** as a compound for further investigation.

As mentioned above, the oxygen atom in the alkoxy group at the 3-position was important for inhibitory activity. An X-ray structural analysis of **11d** suggested an explanation for this finding (Fig. 3).[5] An intramolecular hydrogen bond interaction was observed between the oxygen atom in the 2,2,2-trifluoroethoxy group and the amide hydrogen atom at the C2 side chain. This conformation would not be favored in the C3-ethyl derivative. We speculated that the oxygen atom would be necessary for hydrogen bond formation with the amide, thereby stabilizing the position of the side chain.

We evaluated the in vivo efficacy of **11d** using a Ptc1+/− p53−/− medulloblastoma allograft model in mice in which the Hh pathway was activated. Plasma and tumor concentrations of **11d** after a single oral dose are shown in Figure 4 indicating good tumor permeability and dose linearity. Repeated oral administration of compound **11d** (QD, 14 days) resulted in moderate antitumor activity with a treated/control (T/C) value of 46% at a dose of 1 mg/kg (Fig. 5); complete growth inhibition was observed at 25 mg/kg (T/C = 1%) without excessive accumulation. These data suggest that improved solubility resulted in a dose-dependent PK profile for compound **11d**.

Table 3
Optimization of pyrroro[3,2-*c*]pyridine at the 3-position



Compds	R^4	Gli-luc reporter IC_{50}[a] (nM)	Solubility[b] (μg/mL)	AUC[c] (μgh/mL)
11e	OMe	5.7	63	31.647
11h	Et	95		
11a	OEt	6.9	58	20.678
11b	OCH_2CH_2F	4.9	69	5.149
11c	OCH_2CHF_2	4.4	84	5.404
11d	OCH_2CF_3	4.4	81	28.346

[a] IC_{50} values are the mean of four measurements.
[b] Measured at pH 6.8 (Japanese Pharmacopoeia second fluid).
[c] Cassette dosing at 10 mg/kg, po in mice. AUC: area under the plasma concentration versus time curve from 0 to 8 h.

The PK profiles of **11d** in other species are shown in Table 5. Compound **11d** showed favorable oral bioavailability (*F*), which was 32% in rats and 90% in dogs. Moreover, low drug clearance (CL) and high AUC were observed in dogs. These results suggested that compound **11d** is able to achieve sufficient exposure following oral administration in rats and dogs to enable appropriate toxicological studies.

4. Conclusions

With the aim of improving solubility, chemical modification of the previously developed compound **1** was examined. Decreasing the planarity by reducing the number of aromatic rings led to the identification of the novel pyrrolo[3,2-*c*]pyridine-4-one derivative **11j** which had good solubility. Further optimization of the 3-alkoxy group resulted in discovery of **11d** with good physicochemical profiles and potent activity. Compound **11d** exhibited strong antitumor activity in an in vivo study in Ptc1+/− p53−/− mice bearing medulloblastoma allografts; improved solubility of compound **11d** enabled dose-dependent plasma and tumor concentrations to be achieved in this model. On the basis of the overall pharmaceutical profile, compound **11d** was identified as a potential candidate for clinical development, and a clinical trial of **11d** (TAK-441) is currently ongoing.

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) down-field from internal tetramethylsilane of the indicated organic solution. Peak multiplicities are expressed using the following abbreviations: 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,

Table 4
Pharmacokinetic data of **1** and **11d**

Compd	Mouse PK 10 mg/kg[a]		Mouse PK 100 mg/kg[b]	
	C_{max} (μg/mL)	AUC (μgh/mL)	C_{max} (μg/mL)	AUC (μgh/mL)
1	2.65	12.1	3.63	32.3
11d	5.62	28.3	21.5	206

[a] Cassette dosing, AUC_{0-8h}.
[b] BALB/c-nu/nu mice, AUC_{0-24h}.

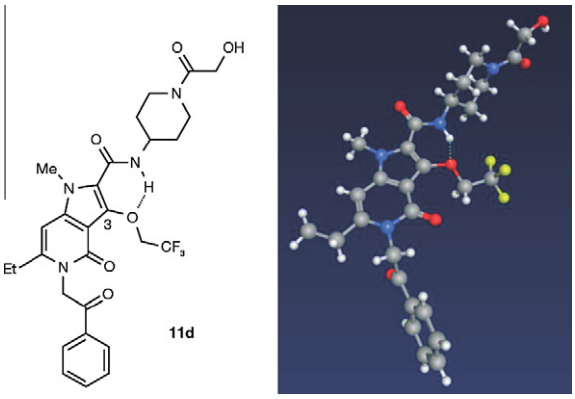


Figure 3. X-ray crystal structure of compound **11d**. The figure was described based on X-ray structural analysis data using MOE Software. Molecular Operating Environment (MOE version 2010.10); Chemical Computing Group, Inc.: Montreal, Quebec, Canada; http://www.chemcomp.com.

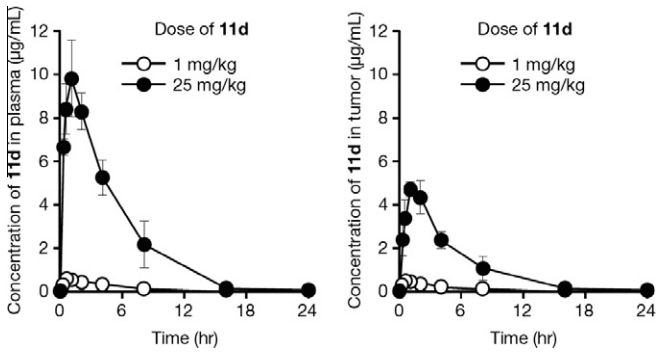


Figure 4. Concentrations of compound **11d** in the plasma and tumor after oral administration at doses of 1 and 25 mg/kg in medulloblastoma allografted mice. Mean ± standard deviation, SD ($n = 3$).

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). Reaction progress was determined by thin layer chromatography (TLC) analysis on silica gel $60F_{254}$ plate (Merck) or NH TLC plates (Fuji Silysia).

5.1. Methyl (2*Z*)-3-aminopent-2-enoate (3a)

A mixture of **2a** (75.0 g, 576 mmol), NH_4OAc (222 g, 2.88 mmol) and MeOH (750 mL) was stirred at room temperature for 3 days. The mixture was concentrated in vacuo. The residue was diluted with water (500 mL) and extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$, concentrated in vacuo, and dried to give the title compound (68.5 g, 92%) as a pale yellow oil. ^{1}H NMR (DMSO-d_6) δ 1.06 (3H, t, $J = 7.6$ Hz), 2.09 (2H, q, $J = 7.6$ Hz), 3.49 (3H, s), 4.34 (1H, s), 6.94 (1H, s), 7.72 (1H, br s).

5.2. Ethyl 2-aminocyclohex-1-ene-1-carboxylate (3b)

In the same manner as in the preparation of **3a**, the title compound (32.3 g, 60%) was obtained as a white solid from **2b** (53.9 g, 0.317 mol). ^{1}H NMR (DMSO-d_6) δ 1.16 (3H, t, $J = 7.1$ Hz), 1.45–1.59 (4H, m), 2.09–2.21 (4H, m), 4.00 (2H, q, $J = 7.1$ Hz), 7.09 (2H, br s).

5.3. Ethyl 6-ethyl-4-hydroxy-2-oxo-1,2-dihydropyridine-3-carboxylate (4a)

A mixture of **3a** (100 g, 774 mmol), diethyl malonate (130 mL, 856 mmol), 20% solution of NaOEt in EtOH (290 g, 852 mmol), EtOH (400 mL), xylene (800 mL) was stirred at 120 °C for 2 h, at 150 °C for 17 h with a Dean–Stark trap. The precipitate was collected by filtration and washed with hexane. The filtered material was dissolved in water (800 mL) and filtered. The filtrate was acidified with 5 M HCl aq at 0 °C. The precipitate was collected by filtration, successively washed with water and hexane/IPE to give the title compound (75.4 g, 46%) as a pale yellow powder. ^{1}H NMR (DMSO-d_6) δ 1.12 (3H, t, $J = 7.6$ Hz), 1.26 (3H, t, $J = 7.1$ Hz), 2.42 (2H, q, $J = 7.6$ Hz), 4.25 (2H, q, $J = 7.1$ Hz), 5.79 (1H, s), 11.37 (1H, br s), 12.56 (1H, s).

5.4. Ethyl 4-hydroxy-2-oxo-1,2,5,6,7,8-hexahydroquinoline-3-carboxylate (4b)

In the same manner as in the preparation of **4a**, the title compound (24.4 g, 58%) was obtained as a yellow solid from **3b** (30.0 g, 0.177 mol). ^{1}H NMR (DMSO-d_6) δ 1.28 (3H, t, $J = 7.1$ Hz), 1.57–1.73 (4H, m), 2.24–2.35 (2H, m), 2.41–2.48 (2H, m), 4.30 (2H, q, $J = 7.1$ Hz), 11.19 (1H, s), 13.49 (1H, s).

5.5. Ethyl 4-chloro-6-ethyl-2-oxo-1,2-dihydropyridine-3-carboxylate (5a)

A mixture of **4a** (15.0 g, 71.0 mmol) and $POCl_3$ (19.9 mL, 213 mmol) was stirred at 80 °C for 30 min. The mixture was concentrated in vacuo and ice water was added to the residue at 0 °C. The resulting solid was collected by filtration and washed with water and AcOEt to give the title compound (9.80 g, 60%) as a white solid. ^{1}H NMR (DMSO-d_6) δ 1.15 (3H, t, $J = 7.5$ Hz), 1.26 (3H, t, $J = 7.1$ Hz), 2.44–2.55 (2H, m), 4.25 (2H, q, $J = 7.1$ Hz), 6.26 (1H, s), 12.28 (1H, s).

5.6. Ethyl 4-chloro-2-oxo-1,2,5,6,7,8-hexahydroquinoline-3-carboxylate (5b)

A mixture of **4b** (26.0 g, 110 mmol) and $POCl_3$ (51.3 mL, 552 mmol) was stirred at 130 °C for 1.5 h. After cooling, the reaction mixture was concentrated in vacuo and ice was added to the residue. The mixture was neutralized with saturated $NaHCO_3$ aq and extracted with AcOEt. The extract was washed with saturated $NaHCO_3$ aq water and brine, dried over $MgSO_4$, and concentrated in vacuo. The residual solid was collected by filtration and washed with hexane/AcOEt to give the title compound (4.65 g, 17%) as a white solid. ^{1}H NMR (DMSO-d_6) δ 1.25 (3H, t, $J = 7.1$ Hz), 1.61–1.74 (4H, m), 2.37–2.45 (2H, m), 2.51–2.57 (2H, m), 4.24 (2H, q, $J = 7.1$ Hz), 12.09 (1H, s).

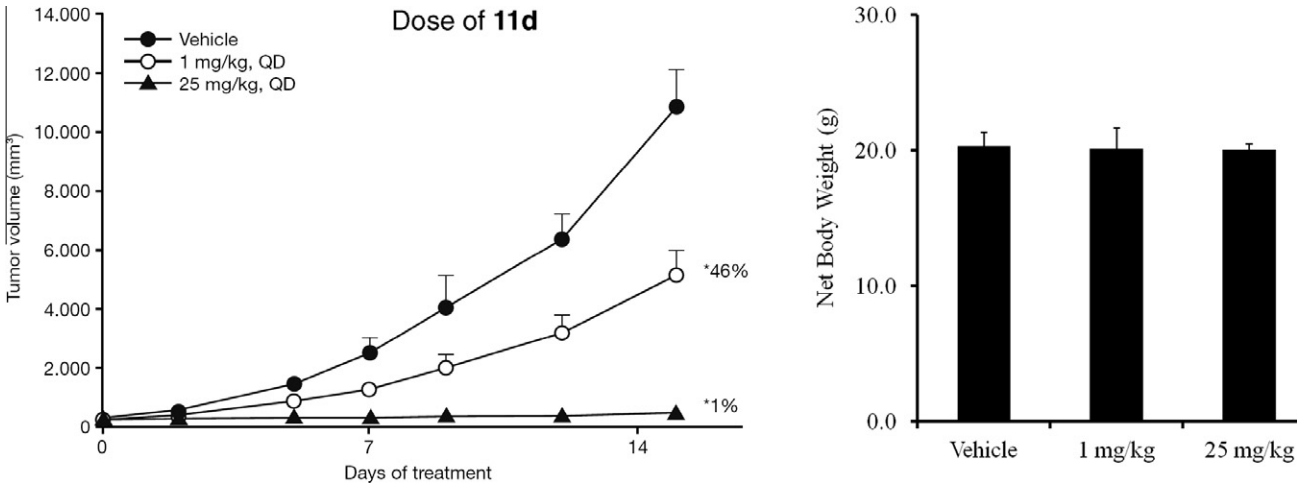


Figure 5. Efficacy of compound **11d** in medulloblastoma in a Ptc1+/− p53−/− mouse allograft model (left) and body weight after 14 days, QD (right). Oral administration of **11d** in mice once daily (QD) for 14 days at 1 mg/kg (▲) or 25 mg/kg (●) compared with vehicle-treated animals (○). Data are shown as mean ± SD ($n = 5$). Antitumor effects were expressed as the ratio of treatment versus control (T/C, %) which was calculated by comparison of the mean change in tumor volume over the treatment period for the control and treated groups. $^{*}P \leqslant 0.025$ by a 1-tailed Shirley-Williams test compared to vehicle control.

Table 5
Pharmacokinetic profile[a] in rats and dogs

	V_{ss} (mL/kg)	CL (mL/h/kg)	$AUC_{0-24h,iv}$ (ng h/mL)	$AUC_{0-24h,po}$ (ng h/mL)	F (%)
Rat[b]	681.6 ± 81.6	397.9 ± 10.1	2532.3 ± 69.1	8031.8 ± 1218.6	31.7
Dog[c]	2181.3 ± 82.8	161.3 ± 35.6	5101.5 ± 685.5	45405.6 ± 5812.0	90.3 ± 8.8

[a] Dose: iv, 1 mg/kg; po, 10 mg/kg.
[b] Each value except for bioavailabilty (F%) represents the mean ± SD of three non-fasted animals. The F value is calculated from mean values of AUC_{0-24h} after oral and intravenous administration.
[c] Each value represents the mean ± SD of four fed animals. The F value is calculated from the individual value of AUC_{0-24h} after oral and intravenous administration for the same animal.

5.7. Ethyl 4-chloro-6-methyl-2-oxo-1,2-dihydropyridine-3-carboxylate (5c)

A mixture of **4c** (3.00 g, 15.2 mmol), $POCl_3$ (7.75 mL, 83.3 mmol), and benzyltriethylammonium chloride (13.8 g, 60.8 mmol) in MeCN (60 mL) was stirred at 40 °C for 30 min and at reflux for 30 min. After cooling, the reaction mixture was concentrated in vacuo, water was added to the residue, and the mixture was extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The residual solid was collected and washed with hexane/AcOEt to give the title compound (1.45 g, 44%) as a white powder. ^{1}H NMR (DMSO-d_6) δ 1.26 (3H, t, J = 7.0 Hz), 2.20 (3H, s), 4.25 (2H, q, J = 7.0 Hz), 6.26 (1H, s), 12.29 (1H, s).

5.8. Ethyl 4-chloro-6-ethyl-2-oxo-1-(2-oxo-2-phenylethyl)-1,2-dihydropyridine-3-carboxylate (6a)

A mixture of **5a** (9.50 g, 41.4 mmol), K_2CO_3 (13.7 g, 99.2 mmol), phenacyl bromide (9.88 g, 49.6 mmol) and DMF (100 mL) was stirred at room temperature 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 (hexane/AcOEt = 9/1 to 3/7) to give the title compound (1.00 g, 7%) as a white powder. ^{1}H NMR (DMSO-d_6) δ 1.14 (3H, t, J = 7.4 Hz), 1.24 (3H, t, J = 7.1 Hz), 2.62 (2H, q, J = 7.4 Hz), 4.25 (2H, q, J = 7.1 Hz), 5.61 (2H, s), 6.44 (1H, s), 7.54–7.66 (2H, m), 7.70–7.79 (1H, m), 8.05–8.13 (2H, m).

5.9. Ethyl 4-chloro-2-oxo-1-(2-oxo-2-phenylethyl)-1,2,5,6,7,8-hexahydroquinoline-3-carboxylate (6b)

Compound **5b** (3.00 g, 11.7 mmol) was added to a suspension of NaH (60% in oil, 516 mg, 12.9 mmol) in DMA (30 mL) and the mixture was stirred at room temperature for 30 min. Phenacyl bromide (2.57 g, 12.9 mmol) was added, and the mixture was stirred at room temperature for 15 h. The mixture was diluted with water, extracted with AcOEt, and washed with water and brine. The organic layer was dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by basic silica gel column chromatography (hexane/AcOEt = 9/1 to AcOEt) to give the title compound (710 mg, 16%) as a white powder. ^{1}H NMR (DMSO-d_6) δ 1.25 (3H, t, J = 7.1 Hz), 1.60–1.80 (4H, m), 2.50–2.65 (4H, m), 4.26 (2H, q, J = 7.1 Hz), 5.66 (2H, s), 7.61 (2H, t, J = 7.4 Hz), 7.74 (1H, t, J = 7.4 Hz), 8.09 (2H, d, J = 7.4 Hz).

5.10. Ethyl 4-chloro-6-methyl-2-oxo-1-(2-oxo-2-phenylethyl)-1,2-dihydropyridine-3-carboxylate (6c)

In the same manner as in the preparation of **6a**, the title compound (3.18 g, 23%) was obtained as a white powder from **5c** (9.00 g, 41.7 mmol). ^{1}H NMR (DMSO-d_6) δ 1.25 (3H, t, J = 7.0 Hz), 2.30 (3H, s), 4.25 (2H, q, J = 7.0 Hz), 5.63 (2H, s), 6.54 (1H, d, J = 0.6 Hz), 7.61 (2H, t, J = 7.5 Hz), 7.72–7.78 (1H, m), 8.07–8.10 (2H, m).

5.11. Ethyl 6-ethyl-3-hydroxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo-[3,2-*c*]pyridine-2-carboxylate (7a)

A mixture of **6a** (7.00 g, 19.3 mmol), sarcosine ethyl ester hydrochloride (5.94 g, 38.7 mmol), Et_3N (29.7 mL, 193 mmol), and EtOH (100 mL) was stirred at reflux for 2 days. After cooling, the mixture was diluted with water and acidified with 5 M HCl aq. The resulting solid was collected and washed with water and hexane/AcOEt to give the title compound (5.70 g, 74%) as a white solid. ^{1}H NMR (DMSO-d_6) δ 1.18 (3H, t, J = 7.4 Hz), 1.31 (3H, t, J = 7.1 Hz), 2.56 (2H, q, J = 7.4 Hz), 3.80 (3H, s), 4.30 (2H, q, J = 7.1 Hz), 5.55 (2H, s), 6.43 (1H, s), 7.60 (2H, t, J = 7.6 Hz), 7.68–7.79 (1H, m), 8.00–8.21 (2H, m), 8.90 (1H, s).

5.12. Ethyl 3-hydroxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5,6,7,8,9-hexahydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxylate (7b)

A mixture of the compound **6b** (270 mg, 0.724 mmol), sarcosine ethyl ester hydrochloride (222 mg, 1.45 mmol), Et_3N (1.00 mL, 7.24 mmol), and EtOH (5 mL) was stirred at reflux for 2 days. After cooling, water (10 mL) was added to the mixture and the resulting solid was collected. This was used for the next reaction without further purification.

5.13. Ethyl 3-hydroxy-1,6-dimethyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]pyridine-2-carboxylate (7c)

In the same manner as in the preparation of **7a**, the title compound (1.50 g, 68%) was obtained as a white powder from **6c** (2.00 g, 5.99 mmol). ^{1}H NMR (DMSO-d_6) δ 1.31 (3H, t, J = 7.1 Hz), 2.26 (3H, s), 3.77 (3H, s), 4.30 (2H, q, J = 7.1 Hz), 5.57 (2H, s), 6.52 (1H, s), 7.54–7.67 (2H, m), 7.69–7.79 (1H, m), 8.03–8.18 (2H, m), 8.90 (1 H, s).

5.14. Ethyl 3-ethoxy-6-ethyl-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]pyridine-2-carboxylate (8a)

K_2CO_3 (10.6 g, 76.8 mmol) and Et_2SO_4 (4.03 mL, 30.1 mmol) were added to a solution of **7a** (9.80 g, 25.6 mmol) in acetone (196 mL) and the mixture was refluxed for 1 h. Et_2SO_4 (4.03 mL, 30.1 mmol) and acetone (70 mL) were added, and the mixture was refluxed for 17 h. The mixture was diluted with water (400 mL) and the resulting precipitate was collected by filtration and washed with water and hexane/AcOEt to give the title compound (8.58 g, 82%) as a white solid. ^{1}H NMR (DMSO-d_6) δ 1.13–1.27 (6H, m), 1.31 (3H, t, J = 7.1 Hz), 2.57 (2H, q, J = 7.3 Hz), 3.84 (3H, s), 4.15 (2H, q, J = 7.1 Hz), 4.26 (2H, q, J = 7.1 Hz), 5.57 (2H, s), 6.49 (1H, s), 7.56–7.66 (2H, m), 7.69–7.77 (1H, m), 8.07–8.16 (2H, m).

5.15. Ethyl 6-ethyl-3-(2-fluoroethoxy)-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]pyridine-2-carboxylate (8b)

2-Fluroroethyl iodide (512 mg, 2.94 mmol) was added to a mixture of **7a** (750 mg, 1.96 mmol) and DBU (0.440 mL, 2.94 mmol) in DMF (10 mL) and the mixture was stirred at room temperature for 15 h. The mixture was diluted with water and extracted with AcOEt. The organic layer was washed with water and brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by basic silica gel column chromatography (AcOEt) to give the title compound (649 mg, 77%) as a white solid. ^{1}H NMR (DMSO-d_6) δ 1.19 (3H, t, J = 7.3 Hz), 1.30 (3H, t, J = 7.1 Hz), 2.58 (2H, q, J = 7.3 Hz), 3.85 (3H, s), 4.26 (2H, q, J = 7.1 Hz), 4.32–4.36 (1H, m), 4.42–4.47 (1H, m), 4.53–4.59 (1H, m), 4.69–4.75 (1H, m), 5.58 (2H, s), 6.51 (1H, s), 7.61 (2H, t, J = 7.6 Hz), 7.70–7.77 (1H, m), 8.08–8.14 (2H, m).

5.16. Ethyl 3-(2,2-difluoroethoxy)-6-ethyl-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo-[3,2-*c*]pyridine-2-carboxylate (8c)

2,2-Difluoroethyl trifluoromethanesulfonate (336 mg, 1.57 mmol) was added to a mixture of **7a** (500 mg, 1.31 mmol) and Cs_2CO_3 (554 mg, 1.70 mmol) in DMF (5 mL) and the mixture was stirred at room temperature for 2 h. The mixture was diluted with water (15 mL) and the precipitate was collected by filtration. The collected material was washed with water, EtOH and Et_2O, and dried in vacuo to give the title compound (0.45 g, 77%) as a white solid. ^{1}H NMR (DMSO-d_6) δ 1.19 (3H, t, J = 7.4 Hz), 1.30 (3H, t, J = 7.2 Hz), 2.59 (2H, q, J = 7.4 Hz), 3.86 (3H, s), 4.26 (2H, q, J = 7.2 Hz), 4.42 (2H, td, J = 14.9, 3.9 Hz), 5.61 (2H, s), 6.27 (1H, tt, J = 54.9, 3.9 Hz), 6.54 (1H, s), 7.61 (2H, t, J = 7.5 Hz), 7.74 (1H, t, J = 7.5 Hz), 8.10–8.13 (2H, m).

5.17. Ethyl 6-ethyl-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-3-(2,2,2-trifluoroethoxy)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]pyridine-2-carboxylate (8d)

In the same manner as in the preparation of **8c**, the title compound (7.81 g, 86%) was obtained as a white solid from **7a** (7.47 g, 19.5 mmol). ^{1}H NMR (DMSO-d_6) δ 1.19 (3H, t, J = 7.4 Hz), 1.29 (3H, t, J = 7.1 Hz), 2.59 (2H, q, J = 7.4 Hz), 3.86 (3H, s), 4.26 (2H, q, J = 7.1 Hz), 4.83 (2H, q, J = 9.3 Hz), 5.61 (2H, s), 6.56 (1H, s), 7.61 (2H, t, J = 7.6 Hz), 7.69–7.79 (1H, m), 8.06–8.18 (2H, m).

5.18. Ethyl 6-ethyl-3-methoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]pyridine-2-carboxylate (8e)

In the same manner as in the preparation of **8b**, the title compound (156 mg, 23%) was obtained as a white powder from **7a** (650 mg, 1.70 mmol). ^{1}H NMR (DMSO-d_6) δ 1.19 (3H, t, J = 7.3 Hz), 1.30 (3H, t, J = 7.1 Hz), 2.58 (2H, q, J = 7.4 Hz), 3.84 (3H, s), 3.85 (3H, s), 4.27 (2H, q, J = 7.0 Hz), 5.58 (2H, s), 6.50 (1H, s), 7.61 (2H, t, J = 7.6 Hz), 7.67–7.82 (1H, m), 8.03–8.19 (2H, m).

5.19. Ethyl 6-ethyl-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-3-{[(trifluoromethyl) sulfonyl]oxy}-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]pyridine-2-carboxylate (8f)

A mixture of **7a** (300 mg, 0.784 mmol), Tf_2O (158 μL, 0.941 mmol), and pyridine (6 mL) was stirred at 60 °C for 3 h under N_2 atmosphere. The mixture was diluted with water (50 mL) and extracted with AcOEt (100 mL). The extract was washed with brine (50 mL), dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/AcOEt = 1/1 to AcOEt) to give the title compound (239 mg, 59%) as a pale yellow powder. ^{1}H NMR (DMSO-d_6) δ 1.20 (3H, t, J = 7.3 Hz), 1.33 (3H, t, J = 7.2 Hz), 2.60 (2H, q, J = 7.3 Hz), 3.95 (3H, s), 4.34 (2H, q, J = 7.2 Hz), 5.64 (2H, s), 6.64 (1H, s), 7.57–7.68 (2H, m), 7.70–7.81 (1H, m), 8.07–8.19 (2H, m).

5.20. Ethyl 3-ethenyl-6-ethyl-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo-[3,2-*c*]pyridine-2-carboxylate (8g)

A mixture of **8f** (218 mg, 0.424 mmol), vinyltributyltin (328 μL, 1.28 mmol), $Pd(PPh_3)_4$ (148 mg, 0.128 mmol) and DMF

(5.9 mL) was stirred at 100 °C for 3 h under Ar atmosphere. The mixture was diluted with water (50 mL) and extracted with AcOEt (100 mL). The extract was washed with brine (50 mL), dried over $MgSO_4$, and concentrated in vacuo. The insoluble material was removed by filtration and the filtrate was concentrated in vacuo. The precipitate was collected by filtration and washed with IPE to give the title compound (120 mg, 72%) as a grey solid. ^{1}H NMR (DMSO-d_6) δ 1.20 (3H, t, J = 7.3 Hz), 1.33 (3H, t, J = 7.1 Hz), 2.61 (2H, q, J = 7.3 Hz), 3.85 (3H, s), 4.33 (2H, q, J = 7.1 Hz), 5.29–5.43 (1H, m), 5.59 (2H, s), 6.41–6.59 (2H, m), 7.23 (1H, dd, J = 17.7, 11.8 Hz), 7.55–7.67 (2H, m), 7.68–7.81 (1H, m), 8.01–8.20 (2H, m).

5.21. Ethyl 3,6-diethyl-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]-pyridine-2-carboxylate (8h)

A mixture of **8g** (1.33 g, 3.40 mmol), 10% Pd-C (270 mg) and THF (50 mL) /MeOH (25 mL) was stirred at room temperature for 7 h under H_2 atmosphere. The catalyst was removed by filtration and the filtrate was concentrated in vacuo. The precipitate was collected by filtration to give the title compound (1.35 g, 100%) as a white solid. ^{1}H NMR (DMSO-d_6) δ 1.09 (3H, t, J = 7.2 Hz), 1.19 (3H, t, J = 7.5 Hz), 1.33 (3H, t, J = 7.2 Hz), 2.58 (2H, q, J = 7.2 Hz), 3.07–3.19 (2H, m), 3.87 (3H, s), 4.30 (2H, q, J = 7.2 Hz), 5.59 (2H, s), 6.48 (1H, s), 7.58–7.64 (2H, m), 7.70–7.77 (1H, m), 8.08–8.14 (2H, m).

5.22. Ethyl 3-methoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5,6,7,8,9-hexahydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxylate (8i)

In the same manner as in the preparation of **8a**, the title compound (34.0 mg, 11% in two steps) was obtained as a white powder from **7b**. ^{1}H NMR (DMSO-d_6) δ 1.31 (3H, t, J = 7.1 Hz), 1.60–1.77 (4H, m), 2.45–2.55 (2H, m), 2.94–3.02 (2H, m), 3.81 (3H, s), 4.03 (3H, s), 4.27 (2H, q, J = 7.1 Hz), 5.62 (2H, s), 7.61 (2H, t, J = 7.5 Hz), 7.73 (1H, t, J = 7.5 Hz), 8.10 (2H, d, J = 7.5 Hz).

5.23. Ethyl 3-methoxy-1,6-dimethyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo-[3,2-*c*]pyridine-2-carboxylate (8j)

In the same manner as in the preparation of **8b**, the title compound (390 mg, 26%) was obtained as a white powder from **7c** (1.45 g, 3.94 mmol). ^{1}H NMR (DMSO-d_6) δ 1.30 (3H, t, J = 7.1 Hz), 2.28 (3H, s), 3.81 (3H, s), 3.86 (3H, s), 4.27 (2H, q, J = 7.1 Hz), 5.60 (2H, s), 6.59 (1H, s), 7.54–7.66 (2H, m), 7.69–7.79 (1H, m), 8.05–8.16 (2H, m).

5.24. 3-Ethoxy-6-ethyl-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]-pyridine-2-carboxylic acid (9a)

8 M NaOH aq (42.9 mL) was added to a solution of **8a** (8.58 g, 20.9 mmol) in EtOH (257 mL) and the mixture was stirred at 60 °C for 30 min. The mixture was diluted with water and acidified with 5 M HCl aq The resulting precipitate was collected by filtration, and washed with water and Et_2O to give the title compound (7.12 g, 89%) as a beige powder. ^{1}H NMR (DMSO-d_6) δ 1.12–1.27 (6H, m), 2.57 (2H, q, J = 7.4 Hz), 3.84 (3H, s), 4.16 (2H, q, J = 7.1 Hz), 5.58 (2H, s), 6.48 (1H, s), 7.55–7.66 (2H, m), 7.68–7.78 (1H, m), 8.05–8.19 (2H, m), 12.41 (1H, br s).

The following compounds **9b–j** were prepared in a same manner similar to that described for **9a**.

5.25. 6-Ethyl-3-(2-fluoroethoxy)-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo-[3,2-*c*]pyridine-2-carboxylic acid (9b)

Yield 56%, white powder. ^{1}H NMR (DMSO-d_6) δ 1.19 (3H, t, J = 7.4 Hz), 2.58 (2H, q, J = 7.4 Hz), 3.85 (3H, s), 4.32–4.36 (1H, m), 4.42–4.47 (1H, m), 4.54–4.59 (1H, m), 4.70–4.74 (1H, m), 5.58 (2H, s), 6.50 (1H, s), 7.61 (2H, t, J = 7.6 Hz), 7.70–7.77 (1H, m), 8.09–8.15 (2H, m), 12.48 (1H, br s).

5.26. 3-(2,2-Difluoroethoxy)-6-ethyl-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]pyridine-2-carboxylic acid (9c)

Yield 83%, white powder. ^{1}H NMR (DMSO-d_6) δ 1.19 (3H, t, J = 7.2 Hz), 2.58 (2H, q, J = 7.3 Hz), 3.85 (3H, s), 4.40 (2H, td, J = 14.6, 1.2 Hz), 5.60 (2H, s), 6.26 (1H, tt, J = 55.0, 3.9 Hz), 6.77 (1H, s), 7.58–7.76 (3H, m), 8.12 (2H, d, J = 7.5 Hz), 12.50–12.70 (1H, m).

5.27. 6-Ethyl-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-3-(2,2,2-trifluoroethoxy)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]pyridine-2-carboxylic acid (9d)

Yield 93%, white powder. ^{1}H NMR (DMSO-d_6) δ 1.19 (3H, t, J = 7.4 Hz), 2.58 (2H, q, J = 7.4 Hz), 3.86 (3H, s), 4.82 (2H, q, J = 9.2 Hz), 5.61 (2H, s), 6.54 (1H, s), 7.61 (2H, t, J = 7.6 Hz), 7.70–7.77 (1H, m), 8.07–8.16 (2H, m), 12.74 (1H, br s).

5.28. 6-Ethyl-3-methoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo-[3,2-*c*]-pyridine-2-carboxylic acid (9e)

Yield 79%, white powder. ^{1}H NMR (DMSO-d_6) δ 1.19 (3H, t, J = 7.3 Hz), 2.57 (2H, q, J = 7.3 Hz), 3.84 (3H, s), 3.85 (3H, s), 5.58 (2H, s), 6.48 (1H, s), 7.61 (2H, t, J = 7.6 Hz), 7.67–7.86 (1H, m), 7.97–8.31 (2H, m), 12.51 (1H, br s).

5.29. 3,6-Diethyl-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]-pyridine-2-carboxylic acid (9h)

Yield 99%, white powder. ^{1}H NMR (DMSO-d_6) δ 1.07 (3H, t, J = 7.3 Hz), 1.19 (3H, t, J = 7.4 Hz), 2.57 (2H, q, J = 7.4 Hz), 3.11 (2H, q, J = 7.3 Hz), 3.87 (3H, s), 5.58 (2H, s), 6.46 (1H, s), 7.56–7.66 (2H, m), 7.68–7.78 (1H, m), 8.05–8.17 (2H, m), 12.80 (1H, br s).

5.30. 3-Methoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5,6,7,8,9-hexahydro-1*H*-pyrrolo-[3,2-*c*]-quinoline-2-carboxylic acid (9i)

Yield 23%, white powder. ^{1}H NMR (DMSO-d_6) δ 1.67 (4H, m), 2.44–2.52 (2H, m), 2.94–3.02 (2H, m), 3.81 (3H, s), 4.05 (3H, s), 5.62 (2H, s), 7.56–7.65 (2H, m), 7.70–7.78 (1H, m), 8.10 (2H, d, J = 7.6 Hz), 12.56 (1H, br s).

5.31. 3-Methoxy-1,6-dimethyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]-pyridine-2-carboxylic acid (9j)

Yield 89%, white powder. ^{1}H NMR (DMSO-d_6) δ 2.28 (3H, s), 3.81 (3H, s), 3.86 (3H, s), 5.60 (2H, s), 6.58 (1H, s), 7.61 (2H, t, J = 7.4 Hz), 7.74 (1H, t, J = 7.3 Hz), 8.11 (2H, d, J = 7.7 Hz), 12.50 (1H, br s).

5.32. 3-Methoxy-1,6-dimethyl-4-oxo-5-(2-oxo-2-phenylethyl)-*N*-piperidin-4-yl-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]pyridine-2-carboxamide hydrochloride (10a)

A mixture of **9j** (290 mg, 0.818 mmol), EDC (236 mg, 1.23 mmol), HOBt (166 mg, 1.23 mmol), and DMF (5 mL) was

stirred at room temperature for 15 h, after which 4-amino-1-Boc-piperidine (212 mg, 1.06 mmol) was added and the mixture was stirred at room temperature for 15 h. The mixture was diluted with water and extracted with AcOEt. The organic layer was washed with water and brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by basic silica gel column chromatography (AcOEt) to give *tert*-butyl 4-({[3-methoxy-1,6-dimethyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]pyridin-2-yl]carbonyl}amino)piperidine-1-carboxylate. 4 M HCl in AcOEt (4 mL) was added to a solution of the compound obtained above in AcOEt (4 mL) and the mixture was stirred at room temperature for 2 h. The precipitated solid was collected by filtration, and washed with AcOEt to give the title compound (240 mg, 62%) as a white powder. ^{1}H NMR (DMSO-d_6) δ 1.61–1.84 (2H, m), 1.95–2.11 (2H, m), 2.28 (3H, s), 2.92–3.12 (2H, m), 3.20–3.32 (2H, m), 3.83 (3H, s), 3.94–4.15 (4H, m), 5.62 (2H, s), 6.59 (1H, s), 7.61 (2H, t, J = 7.6 Hz), 7.68–7.81 (2H, m), 8.04–8.19 (2H, m), 8.64 (1H, br s.), 8.87 (1H, br s).

5.33. 6-Ethyl-3-methoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-*N*-piperidin-4-yl-4,5 -dihydro-1*H*-pyrrolo[3,2-*c*]pyridine-2-carboxamide hydrochloride (10b)

In the same manner as in the preparation of **10a**, the title compound (97.2 mg, 74%) was obtained as a white powder from **9e** (100 mg, 0.271 mmol). ^{1}H NMR (DMSO-d_6) δ 1.19 (3H, t, J = 7.3 Hz), 1.66–1.85 (2H, m), 1.97–2.09 (2H, m), 2.58 (2H, q, J = 7.4 Hz), 2.95–3.10 (2H, m), 3.19–3.30 (2H, m), 3.87 (3H, s), 3.96–4.14 (4H, m), 5.60 (2H, s), 6.49 (1H, s), 7.55–7.66 (2H, m), 7.69–7.79 (2H, m), 8.12 (2H, d, J = 7.7 Hz), 8.92 (2H, br s).

5.34. 3-Ethoxy-6-ethyl-N-[1-(hydroxyacetyl)piperidin-4-yl]-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]pyridine-2-carboxamide (11a)

EDC (55.6 mg, 0.290 mmol) at 0 °C was added to a mixture of the compound **10a** (74.0 mg, 0.194 mmol), **13** (49.1 mg, 0.252 mmol), HOBt (39.2 mg, 0.290 mmol), and Et_3N (34.9 mL, 0.252 mmol) in DMF (3 mL) and the mixture was stirred at room temperature for 15 h. The reaction mixture was diluted with water and extracted twice with AcOEt. The combined extract was washed with water and saturated brine, dried over $MgSO_4$, and concentrated in vacuo. The residue was purified by basic silica gel column chromatography (AcOEt) and the obtained solid was recrystallized from AcOEt to give the title compound (61.2 mg, 60%) as white crystals; mp 204 °C. ^{1}H NMR (DMSO-d_6) δ 1.13–1.29 (6H, m), 1.32–1.56 (2H, m), 1.83–1.96 (2H, m), 2.57 (2H, q, J = 7.4 Hz), 2.80–2.94 (1H, m), 3.04–3.19 (1H, m), 3.59–3.72 (1H, m), 3.90 (3H, s), 3.95–4.14 (3H, m), 4.17–4.27 (1H, m), 4.34 (2H, q, J = 7.2 Hz), 4.51 (1H, t, J = 5.4 Hz), 5.59 (2H, s), 6.49 (1H, s), 7.56–7.65 (2H, m), 7.66–7.77 (2H, m), 8.07–8.14 (2H, m). Anal. Calcd for $C_{28}H_{34}N_4O_6$: C 64.35; H 6.56; N 10.72. Found: C, 64.20; H, 6.52; N, 10.61. LC–MS: m/z = 523 (MH^+).

The following compounds **11b**–**d**, **h**, **i** were prepared in a same manner similar to that described for **11a**.

5.35. 6-Ethyl-3-(2-fluoroethoxy)-*N*-[1-(hydroxyacetyl)piperidin-4-yl]-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]pyridine-2-carboxamide (11b)

Yield 80%, white crystals; mp 208 °C (recrystallized from AcOEt/THF). ^{1}H NMR (DMSO-d_6) δ 1.19 (3H, t, J = 7.4 Hz), 1.26–1.48 (2H, m), 1.87 (2H, s), 2.59 (2H, q, J = 7.4 Hz), 2.76–2.91 (1H, m), 3.01–3.17 (1H, m), 3.57–3.73 (1H, m), 3.92 (3H, s), 3.97–4.13 (3H, m), 4.18–4.32 (1H, m), 4.45–4.81 (5H, m), 5.59 (2H, s), 6.49–6.56 (1H, m), 7.55–7.66 (3H, m), 7.69–7.78 (1H, m), 8.06–8.16 (2H, m). Anal. Calcd for $C_{28}H_{33}FN_4O_6$: C, 62.21; H, 6.15; N, 10.36. Found: C, 62.03; H, 6.24; N, 10.18. LC–MS: m/z = 541 (MH^+).

5.36. 3-(2,2-Difluoroethoxy)-6-ethyl-*N*-[1-(hydroxyacetyl)piperidin-4-yl]-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]pyridine-2-carboxamide (11c)

Yield 65%, white crystals; mp 188 °C (recrystallized from acetone/H_2O). ^{1}H NMR (DMSO-d_6) δ 1.19 (3H, t, J = 7.4 Hz), 1.20–1.50 (2H, m), 1.90 (2H, d, J = 9.9 Hz), 2.59 (2H, q, J = 7.4 Hz), 2.83 (1H, t, J = 12.2 Hz), 3.09 (1H, t, J = 11.7 Hz), 3.68 (1H, d, J = 12.9 Hz), 3.90 (3H, s), 3.95–4.20 (3H, m), 4.26 (1H, d, J = 11.7 Hz), 4.51 (1H, t, J = 5.4 Hz), 4.67 (2H, td, J = 16.1, 3.0 Hz), 5.23 (1H, s), 5.61 (2H, s), 6.33 (1H, tt, J = 54.2, 3.0 Hz), 7.49 (1H, d, J = 7.8 Hz), 7.61 (2H, t, J = 7.5 Hz), 7.73 (1H, t, J = 7.5 Hz), 8.10–8.12 (2H, m). Anal. Calcd for $C_{28}H_{32}F_2N_4O_6$: C, 60.21; H, 5.77; N, 10.03. Found: C, 60.23; H, 5.78; N, 10.01. LC–MS: m/z = 559 (MH^+).

5.37. 6-Ethyl-*N*-[1-(hydroxyacetyl)piperidin-4-yl]-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-3-(2,2,2-trifluoroethoxy)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]pyridine-2-carboxamide (11d)

Yield 76%, white crystals; mp 169 °C (recrystallized from EtOH/H_2O). ^{1}H NMR (DMSO-d_6) δ 1.19 (3H, t, J = 7.3 Hz), 1.24–1.51 (2H, m), 1.79–1.98 (2H, m), 2.59 (2H, q, J = 7.3 Hz), 2.75–2.94 (1H, m), 2.98–3.20 (1H, m), 3.57–3.77 (1H, m), 3.86 (3H, s), 3.94–4.17 (3H, m), 4.17–4.38 (1H, m), 4.53 (1H, t, J = 5.3 Hz), 5.05 (2H, q, J = 9.3 Hz), 5.63 (2H, s), 6.54 (1H, s), 7.51 (1H, d, J = 7.7 Hz), 7.55–7.67 (2H, m), 7.68–7.82 (1H, m), 8.01–8.21 (2H, m). Anal. Calcd for $C_{28}H_{31}F_3N_4O_6$: C, 58.33; H, 5.42; N, 9.72. Found: C, 58.32; H, 5.55; N, 9.63. LC–MS: m/z = 577 (MH^+).

5.38. 3,6-Diethyl-*N*-[1-(hydroxyacetyl)piperidin-4-yl]-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]pyridine-2-carboxamide (11h)

Yield 47%, white crystals; mp 141 °C (recrystallized from hexane/AcOEt). ^{1}H NMR (DMSO-d_6) δ 1.06 (3H, t, J = 7.2 Hz), 1.18 (3H, t, J = 7.3 Hz), 1.30–1.54 (2H, m), 1.81–1.93 (2H, m), 2.54–2.63 (2H, m), 2.78–2.92 (3H, m), 3.01–3.17 (1H, m), 3.59–3.73 (4H, m), 3.99–4.16 (3H, m), 4.20–4.31 (1H, m), 4.51 (1H, t, J = 5.5 Hz), 5.58 (2H, s), 6.42 (1H, s), 7.56–7.65 (2H, m), 7.69–7.77 (1H, m), 8.07–8.15 (2H, m), 8.21 (1H, d, J = 7.4 Hz). Anal. Calcd for $C_{28}H_{34}N_4O_5 \cdot 0.5$AcOEt: C, 65.44; H, 6.96; N, 10.17. Found: C, 65.46; H, 7.01; N, 10.27. LC–MS: m/z = 507 (MH^+).

5.39. *N*-[1-(Hydroxyacetyl)piperidin-4-yl]-3-methoxy-1-methyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5,6,7,8,9-hexahydro-1*H*-pyrrolo[3,2-*c*]quinoline-2-carboxamide (11i)

Yield 76%, colorless oil. ^{1}H NMR (DMSO-d_6) δ 1.22–1.25 (2H, m), 1.35–1.56 (2H, m), 1.60–1.74 (4H, m), 1.80–1.92 (2H, m), 2.83–3.22 (4H, m), 3.59–3.70 (1H, m), 3.90 (3H, s), 3.98–4.12 (7H, m), 4.51 (1H, t, J = 5.4 Hz), 5.63 (2H, s), 7.56–7.65 (2H, m), 7.69–7.82 (2H, m), 8.10 (2H, d, J = 8.3 Hz). LC–MS: m/z = 535 (MH^+).

5.40. *N*-[1-(Hydroxyacetyl)piperidin-4-yl]-3-methoxy-1,6-dimethyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]pyridine-2-carboxamide (11j)

Acetoxyacetyl chloride (61.4 μL, 0.571 mmol) at 0 °C was added to a mixture of **10a** (225 mg, 0.476 mmol) and Et_3N (198 μL, 1.43 mmol) in THF (5 mL) and the mixture was stirred at room temperature for 15 h. The mixture was diluted with AcOEt, washed with water and brine, and dried over $MgSO_4$. After removal of

$MgSO_4$ by filtration, the filtrate was concentrated in vacuo. The residue was purified by basic silica gel column chromatography (AcOEt) to give 2-[4-({[3-methoxy-1,6-dimethyl-4-oxo-5-(2-oxo-2-phenylethyl)-4,5-dihydro-1*H*-pyrrolo[3,2-*c*]-pyridin-2-yl]carbonyl}amino)piperidin-1-yl]-2-oxoethyl acetate. 8 M NaOH aq (0.5 mL), THF (2.5 mL) and EtOH (5 mL) were added to a mixture of the compound obtained above and stirred at room temperature for 2 h. The mixture was neutralized with 1 M HCl aq and diluted with water, and extracted with AcOEt/THF. The organic layer was washed with water and brine and dried over $MgSO_4$. After removal of $MgSO_4$ by filtration, the filtrate was concentrated in vacuo. The residue was purified by basic silica gel column chromatography (AcOEt) to give the title compound (156 mg, 66%) as a white solid; mp 237 °C (recrystallized from hexane/AcOEt/THF). ^{1}H NMR (DMSO-d_6) δ 1.32–1.61 (2H, m), 1.79–1.93 (2H, m), 2.28 (3H, s), 2.82–2.98 (1H, m), 3.04–3.20 (1H, m), 3.57–3.71 (1H, m), 3.86 (3H, s), 3.96–4.25 (7H, m), 4.50 (1H, d, J = 5.3 Hz), 5.61 (2H, s), 6.58 (1H, s), 7.55–7.67 (3H, m), 7.69–7.78 (1H, m), 8.07–8.16 (2H, m). Anal. Calcd for $C_{26}H_{30}N_4O_6$: C, 63.15; H, 6.11; N, 11.33. Found: C, 63.03; H, 6.15; N, 11.07. LC–MS: m/z = 495 (MH^+).

5.41. 6-Ethyl-*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*]pyridine-2-carboxamide (11e)

In the same manner as in the preparation of **11j**, the title compound (61.2 mg, 65%) was obtained as a white powder from **10b** (90.0 mg, 0.185 mmol); mp 146 °C (recrystallized from AcOEt). ^{1}H NMR (DMSO-d_6) δ 1.13–1.23 (3H, m), 1.31–1.59 (2H, m), 1.80–1.94 (2H, m), 2.58 (2H, q, J = 7.4 Hz), 2.83–2.99 (1H, m), 3.04–3.21 (1H, m), 3.59–3.70 (1H, m), 3.89 (3H, s), 3.97–4.26 (7H, m), 4.49 (1H, t, J = 5.4 Hz), 5.59 (2H, s), 6.49 (1H, s), 7.56–7.67 (3H, m), 7.70–7.78 (1H, m), 8.06–8.15 (2H, m). Anal. Calcd for $C_{27}H_{32}N_4O_6$: C, 63.77; H, 6.34; N, 11.02. Found: C, 63.58; H, 6.31; N, 10.87. LC–MS: m/z = 509 (MH^+).

5.42. 2-(4-Aminopiperidin-1-yl)-2-oxoethanol hydrochloride (13)

Acetoxyacetyl chloride (3.10 mL, 28.8 mmol) was added dropwise at 0 °C to a mixture of **12** (4.80 g, 24.0 mmol) and Et_3N (9.96 mL, 71.9 mmol) in THF (50 mL) and the mixture was stirred for 2 h. The mixture was diluted with AcOEt, washed with water and brine, dried over $MgSO_4$, and concentrated in vacuo. The residual solid was collected by filtration and washed with hexane/AcOEt solution. A mixture of the solid obtained, 8 M NaOH aq (5 mL) and EtOH (35 mL) was stirred at room temperature for 2 h. The mixture was acidified with 6 M HCl aq and concentrated in vacuo. The residue was dissolved in AcOEt (30 mL) and 4 M HCl in AcOEt (30 mL) was added. After stirring at room temperature for 6 h, the resulting solid was collected by filtration and washed with AcOEt to give the title compound (3.73 g, 80%) as a white solid. ^{1}H NMR (DMSO-d_6) δ 1.31–1.51 (2H, m), 1.93 (2H, d, J = 12.9 Hz), 2.69 (1H, t, J = 12.2 Hz), 3.00 (1H, t, J = 12.3 Hz), 3.10–3.30 (1H, m), 3.73 (1H, d, J = 13.8 Hz), 4.08 (2H, q, J = 13.9 Hz), 4.33 (1H, d, J = 12.9 Hz), 8.38 (3H, br s).

5.43. Gli-luc reporter assay

NIH3T3/Gli-luc cells were maintained in DMEM containing 10% FBS, 500-μg/mL G418, and 0.1% gentamicin solution (Invitrogen Corp., Carlsbad, CA). The cells were seeded onto collagen-coated 384-well plates at 7.5 10^3cells/well and cultured overnight in 25 μL /well 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 a serially diluted the compounds in 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 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 (0% or 100% inhibition control, respectively (n = 10 wells). The luciferase activities of reporter cells were measured by Bright-Glo™ (Promega Corp., Madison, WI) using the EnVision® plate reader (PerkinElmer, Inc., Waltham, MA).

5.44. In vivo PD assay

The in vivo PD assay was conducted using nude mice bearing human primary pancreatic tumors (PAN-04). The tumor line was established by the Central Institute for Experimental Animals (Kanagawa, Japan). Compound **11d** was administered orally twice daily. Twenty four hours after the first dose, the tumors were excised and treated with RNAlater (Ambion, Foster, CA). Total RNA samples were isolated using RNeasy Mini kit (Qiagen, Valencia, CA), and first strand cDNA samples were prepared using the high capacity cDNA transcription kit (Applied Biosystems, Carlsbad, CA). The primer sets ofqPCR for quantification of stromal Gli1 mRNA (Applied Biosystems, Carlsbad, CA) were as follows: Mm00494645_m1 (mouse Gli1), 4352339E (mouse GAPDH).

5.45. In vivo anti-tumor test

Anti-tumor effects of compounds were evaluated using a mouse medulloblastoma allogeneic transplantation model[6] in which the medulloblastoma spontaneously occurred in the cerebellum of 7–9-week-old Patch 1 (+/−) and p53 (−/−) double mutant mice. Patch 1 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 tumors were subcutaneously transplanted into nude mice from Charles river laboratories (Yokohama, Japan; CAnN.Cg-Foxn1nu/CrlCrlj), and allograft tumors, following several serial passages in vivo, were used for compound testing. To examine the anti-tumor activity of compound **11d**, animals bearing tumors with an average size of 150–250 mm^3 were treated with compound **11d** (0.5% methylcellulose suspension) twice daily for 2 weeks. The 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 $V = (a \times b^2)/2$. The tumor growth rate (T/C %) was calculated as the mean values for [Treated ($V_{end} - V_{start}$)/Control ($V_{end} - V_{start}$)] × 100.

5.46. Solubility determination

Small volumes of the compounds in DMSO were added to the aqueous buffer (pH 6.8). After incubation, precipitates were separated from by filtration through a filter plate. The filtrates were analyzed for the amount of compound in solution by HPLC analysis.

5.47. Pharmacokinetic studies

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

5.48. Pharmacokinetic profile of rats and dogs

Test compounds were administered to fed rats and dogs. Plasma samples were collected after oral (10 mg/kg) and intravenous (1 mg/kg) administration and were deproteinized with methanol containing an internal standard. After centrifugation, the supernatant was diluted with a mixture of 0.01 mol/L ammonium formate solution and [MeCN/formic acid (100:0.2, v/v)] (7:3, v/v) and centrifuged again. The compound concentrations in the supernatant were measured by LC–MS/MS.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.07.034.

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