A Convenient Synthesis and Biological Research of Novel 5,6,7,8-Tetrahydro-1,6-naphthyridin-2(1*H*)-one Derivatives Hydrochloride as Cytotoxic Agents

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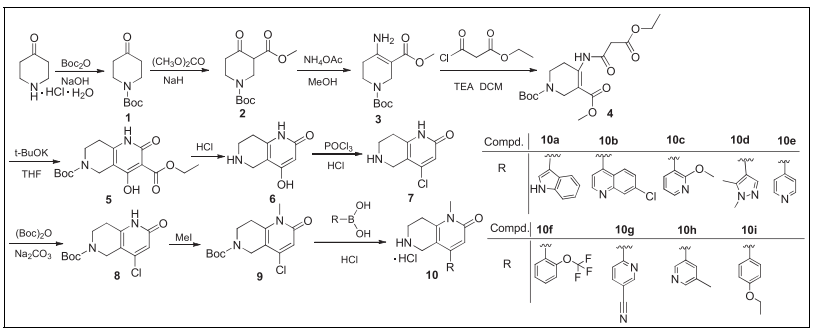


A series of 5,6,7,8-tetrahydro-1,6-naphthyridin-2(1*H*)-one derivatives hydrochloride were obtained using a convenient and mild method from 4-piperidone monohydrate hydrochloride. The newly synthesized compounds and their derivatives were characterized by ^{1}H NMR, ^{13}C NMR, and high-resolution mass spectrometry. Furthermore, cytotoxicity *in vitro* of the synthesized compounds were screened using MTT or CCK8 assay. The results showed that some of the compounds showed potential antitumor activity. Among of them, compound **10a** had effects against tumor cells (MOLM-13), and the half maximal inhibitory concentration value was 76 μmol/L.

INTRODUCTION

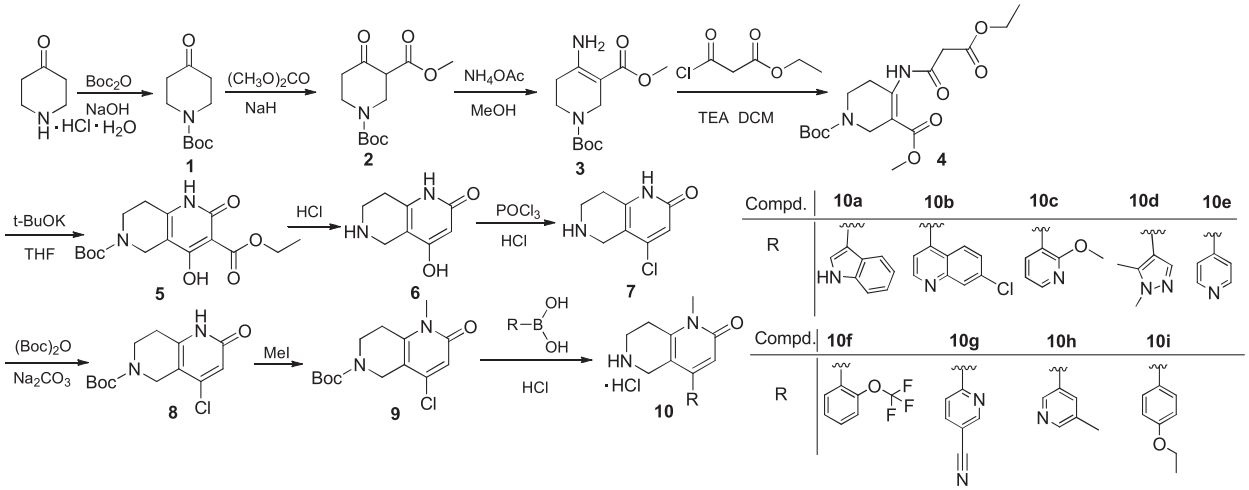
Nitrogen is the most favorite heteroelement in life science research, and heterocyclic compounds containing N attract great interest of the researchers [1–5]. Naphthyridine is an important member of N-containing compounds, and its derivatives have gained special attention of researchers on account of their variety of interesting biological activities in medicinal chemistry. Among naphthyridine derivatives, 1,8-naphthyridine derivatives are most frequently studied on account of their diverse biological activities including antimicrobial, anti-Alzheimer, and antitumor activities [6–10]. 2,7-Naphthyridine derivatives are potent kinase inhibitors [11–14]. 1,6-Naphthyridine derivatives are Syk inhibitors, and some researchers devoted to their synthesis [15–18]. As members of the naphthalene derivative, Naphthyridine-ketone derivatives and tetrahydro-naphthyridine derivatives also arouse the attention because of their different activities [19–24].

In order to fine more new type of naphthyridine derivatives and in the search for new bioactive compound, a series of novel 5,6,7,8-tetrahydro-1,6-naphthyridin-2(1*H*)-one derivatives were designed, synthesized, and characterized. Furthermore, their cytotoxic activity against cancer cell lines (HepG2 and MOLM-13) were evaluated. The results revealed that some of the compounds showed potential antitumor activity.

RESULTS AND DISCUSSION

The synthetic strategy for the preparation of the target compounds is illustrated in Scheme 1. The nitrogen atom of 4-piperidone monohydrate hydrochloride was protected by di-tert-butyl dicarbonate ($(Boc)_2O$) to give **1** according to the literature, and then compound **2** was synthesized in the presence of sodium hydride. Dehydration condensation reaction occurred between compound **2** and ammonium acetate to give **3**, followed by acylation reaction of **3** to give **4**. The active methylene group of **4** was acylated in the presence of *t*-BuOK, followed by the deprotection of N atom that gave the desired key intermediate **6**. Then the hydroxyl group was

Scheme 1. Synthesis of 5,6,7,8-tetrahydro-1,6-naphthyridin-2(1*H*)-one derivatives (**10a–i**).



replaced by chlorine to give **7**. After protection and methylation of the two N atoms, respectively, nucleophile substitution reaction of **9** with different boronic acid afforded the target compounds **10a–i**.

During the reaction, compounds **3** and **4** could be used in the next step without further purification. The reaction conditions of other steps were all convenient and easy to control. The structures of some key intermediates and all target compounds were confirmed by nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) and high-resolution mass spectrometry (HRMS).

Cytotoxicity of compounds **6** and **10a–i** were determined.

In vitro cytotoxicity of **6** and all target compounds were evaluated using tumor cell lines (HepG2 and MOLM-13) exposed at concentration of 100 μmol/L at 72 h. As indicated in Table 1, the inhibition rate of most of the test compounds exceeded 10%, and most of them showed better activity against MOLM-13. Compound **10a**, containing indole ring at the C-4 position, was the best against MOLM-13 with the inhibition rate of 57%. Compared with **10c**, **10e**, **10g**, and **10h**, which possessing (substituted) pyridine ring at the C-4 position, **10h** is the best. It may be due to the effect of methyl. Compound **10f** was the best against HepG2 with the inhibition rate of 32%.

Table 1

Inhibition rate of **6** and all target compounds towards HepG2 and MOLM-13.

Compd.	Tumor cell inhibition rate (%)	
	HepG2	MOLM-13
6	11	24
10a	12	57
10b	8	31
10c	11	14
10d	7	19
10e	6	16
10f	32	42
10g	4	14
10h	7	27
10i	10	16
DMSO	0	0

Table 2

IC50 of the selected compounds.

Compd.	IC50 (μmol/L ± SD[a])	
	HepG2	MOLM-13
6	-	255.7 ± 11.2
10a	249.4 ± 10.6	76.2 ± 6.8
10b	-	194.4 ± 12.1
10f	187.5 ± 8.1	121.1 ± 15.9
10h	-	205.6 ± 12.3
Doxorubicin[b]	4.7 ± 0.2	0.3 ± 0.1

IC50, half maximal inhibitory concentration; -, not detected.
[a]SD is the standard deviation.
[b]Doxorubicin as a positive control.

Half maximal inhibitory concentration value of selected compounds (**6**, **10a**, **10b**, **10f**, and **10h**) was evaluated with doxorubicin as a positive control, and the date was indicated in Table 2. The results showed that the activities of these compounds were considerably weaker than the positive control doxorubicin, but **10a** had the values for further research because of its better activity towards MOLM-13.

CONCLUSION

In summary, we described a novel, efficient, and simple method for the preparation of 5,6,7,8-tetrahydro-

1,6-naphthyridin-2(1*H*)-one derivatives of potential pharmaceutical interest. The mild and convenient reaction conditions of products make it an interesting process. Our study shows that some of the compounds showed potential antitumor activity. Among of them, compound **10a** had effects against tumor cells (MOLM-13), and the half maximal inhibitory concentration value was 76 μmol/L.

EXPERIMENTAL

Chemistry. Solvents and reagents were obtained from commercial sources and used without further purification. All reactions were monitored by thin-layer chromatography. Melting points were determined with a YUHUA X-3 melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker 400 MHz spectrometer operating at 400.13 and 100.61 MHz, respectively, using DMSO-d_6, $CDCl_3$, or MeOD as solvent and TMS as internal standard. Chemical shifts are expressed as ppm (δ). HRMS were obtained on a Bruker MicrOTOF-Q II mass spectrometer with an ESI source.

Synthesis of* tert-*butyl 4-oxopiperidine-1-carboxylate (compound 1). Compound **1** was prepared according to the previously reported method [25] with m.p. 63–65°C.

Synthesis of 1-*tert-*butyl 3-methyl 4-oxopiperidine-1,3-dicarboxylate (compound 2). Compound **2** was prepared according to the literature procedure [26] with m.p. 32–34°C.

Synthesis of 1-*tert-*butyl 3-methyl 4-amino-5,6-dihydropyridine-1,3(2*H*)-dicarboxylate (compound 3). To a solution of compound **2** (25 g, 0.1 mol) in methanol (300 mL), ammonium acetate (22 g, 0.3 mol) was added. After reaction, methanol was removed *in vacuo*, and water (900 mL) was added to the residue. Extraction was performed using CH_2Cl_2 (300 mL × 3). The combined extracts were washed with water, dried over Na_2SO_4, and concentrated *in vacuo* to provide the crude product **3** in 95% yield as red oil, which could be used in the next step without further purification.

Synthesis of 1-*tert-*butyl 3-methyl 4-(3-ethoxy-3-oxopropanamido)-5,6-dihydropyridine-1,3(2*H*)-dicarboxylate (compound 4). To a solution of compound **3** (25 g, 0.1 mol) in CH_2Cl_2 (250 mL), triethylamine (11 g, 0.11 mol) was added. The mixture was cooled to 10°C, and ethyl 3-chloro-3-oxopropanoate (16 g, 0.105 mol) was added dropwise. The mixture was stirred overnight at room temperature. After that, CH_2Cl_2 (250 mL) was added to dilute the reaction mixture, and the mixture was washed with water. The organic phase was dried over Na_2SO_4 and concentrated *in vacuo* to provide the crude product **4** as red oil. The crude product was used in the next step without further purification.

Synthesis of 6-*tert-*butyl 3-ethyl 4-hydroxy-2-oxo-1,2,7,8-tetrahydro-1,6-naphthyridine-3,6(5*H*)-dicarboxylate (compound 5). To a solution of compound **4** (37 g, 0.1 mol) in THF (400 mL), t-BuOK (23 g, 0.2 mol) was added in batches to control the reaction temperature under 25°C. After the reaction was performed for 1 h, cole water (300 mL) was added to quench the reaction, and 2 mol/L HCl was used to adjust the pH 3. After filtration and vacuum drying, compound **5** was obtained as a white solid in a yield of 91.7%.^{1}H NMR (400 MHz, $CDCl_3$): δ 13.72 (s, 1H), 11.89 (s, 1H), 4.47–4.41 (dd, J_1 = 16.0 Hz, J_2 = 16.0 Hz, 2H), 4.32 (s, 2H), 3.68 (t, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 2H), 2.72 (t, J_1 = 4.0 Hz, J_2 = 4.0 Hz, 2H), 1.49 (s, 9H), 1.44 (t, J_1 = 4.0 Hz, J_2 = 8.0 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.20, 162.70, 160.25, 156.28, 149.12, 105.64, 97.97, 87.95, 81.89, 62.81, 52.75, 28.62, 27.39, 14.52. HRMS ((+)-ESI): *m/z* = 361.1335 (calcd. 361.1370 for $C_{16}H_{22}N_2O_6$, $[M + Na]^+$).

Synthesis of 4-hydroxy-5,6,7,8-tetrahydro-1,6-naphthyridin-2(1*H*)-one (6). Compound **5** (34 g, 0.1 mol) was added in batches to 6 mol/L HCl (200 mL). The mixture was refluxed overnight. After concentrated *in vacuo*, the crude was recrystallized with ether/methol (*V*/V = 5:1), and compound **6** (15 g) was obtained as an off-white solid. ^{1}H NMR (400 MHz, CD_3OD): δ 5.03 (s, 1H), 2.23 (t, J_1 = 8.0 Hz, J_2 = 4.0 Hz, 2H), 1.94 (m, 2H), 1.78 (t, J_1 = 4.0 Hz, J_2 = 8.0 Hz, 2H). ^{13}C NMR (100 MHz, CD_3OD): δ 163.69, 155.97, 147.08, 116.88, 113.02, 82.02, 31.23, 28.60. HRMS ((+)-ESI): *m/z* = 167.0792 (calcd. 167.0815 for $C_8H_{10}N_2O_2$, $[M + H]^+$).

Synthesis of* tert-*butyl 4-chloro-2-oxo-1,2,7,8-tetrahydro-1,6-naphthyridine-6(5*H*)-carboxylate (8). In a closed reaction flask with $POCl_3$ (62.5 g, 0.625 mol), compound **6** (20 g, 0.12 mol) was added in batches. After addition, the mixture was slowly heated to 100°C, and the reaction was performed overnight. The reaction mixture was concentrated *in vacuo*, and the concentrate was added to 1,4-dioxane (100 mL). Subsequently, concentrated hydrochloric acid (100 mL) was added slowly, and the mixture was refluxed for 5 h. The solvent was concentrated *in vacuo*, and ethyl acetate (500 mL) was added to the concentrate, followed by washing with water three times. The combined organic phase was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo* to give compound **7** as brown solid. Compound **7** was added directly to a solution of 1,4-dioxane (200 mL) and water (100 mL), followed by the incremental addition of Na_2CO_3 (31 g, 0.3 mol) and $(Boc)_2O$ (33 g, 0.15 mol). After stirring for 10 h at room temperature, the mixture was filtered. The filtrate was extracted with ethyl acetate, and the organ phase was washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The crude product was recrystallized with methol/water (*V*/V = 4:1) give **8** (24 g) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.54

(s, 1H), 4.37 (s, 2H), 3.70 (s, 2H), 3.67 (t, J_1 = 4.0 Hz, J_2 = 8.0 Hz, 2H), 2.77 (t, J_1 = 4.0 Hz, J_2 = 8.0 Hz, 2H), 1.49 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 164.13, 156.15, 151.56, 148.97, 144.29, 117.71, 112.16, 82.03, 68.15, 28.59, 27.32. HRMS ((+)-ESI): *m/z* = 307.0782 (calcd. 307.0820 for $C_{13}H_{17}ClN_2O_3$, $[M + Na]^+$).

***Synthesis of* tert-*butyl* 4-*chloro*-1-*methyl*-2-*oxo*-1,2,7,8-*tetrahydro*-1,6-*naphthyridine*-6(5H)-*carboxylate* (*9*).** Compound **8** (28 g, 0.1 mol) was added to DMF (300 mL), followed by the addition of Cs_2CO_3 (48 g, 0.15 mol) and of iodo methane (22 g, 0.13 mol). The mixture was stirred at room temperature overnight and quenched by cold water. After that, the mixture was extracted with ethyl acetate (300 mL × 3). The combined organic phase was washed with brine, dried over Na_2SO_4, and evaporated to give the crude of **8**. Pulp refining with ether (100 mL) was used to purify **8**. After filtration and vacuum drying, compound **8** was obtained as a white solid in a yield of 83.2%. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.63 (s, 1H), 4.37 (s, 2H), 3.70 (t, J_1 = 4.0 Hz, J_2 = 8.0 Hz, 2H), 3.49 (s, 3H), 2.73 (t, J_1 = 8.0 Hz, J_2 = 4.0 Hz, 2H), 1.50 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.66, 154.21, 145.21, 134.39, 117.15, 111.02, 103.39, 80.76, 42.63, 30.45, 28.37, 27.31. HRMS ((+)-ESI): *m/z* = 321.0972 (calcd. 321.1009 for $C_9H_{20}N_2O_6$, $[M + Na]^+$).

Synthesis of 10a–i. (General procedure) Compound **9** (10 mmol) was added to THF (60 mL), followed by the addition of 1 mol/L potassium phosphate solution (30 mL), and substituted boronic acid (12 mmol). The mixture was refluxed overnignt and extracted with ethyl acetate (30 mL × 3). The combined organic phase was concentrated, followed by the addition of methanol (30 mL) and HCl/1,4-dioxane (30 mL *V*/V = 1:5). The mixture was stirred at room temperature for 5 h. The reaction mixture was concentrated, followed by pulp refining with ether (30 mL) to purify **10a–i**.

*4-(1*H*-indol-3-yl)-1-methyl-5,6,7,8-tetrahydro-1,6-naphthyridin-2(1H)-one hydrochloride* (***10a***). White solid; yield 72%; ^{1}H NMR (600 MHz, DMSO-d_6): δ 11.76 (s, 1H), 9.57 (s, 2H), 7.61 (d, *J* = 6.0 Hz, 1H), 7.54–7.49 (m, 1H), 7.21–7.18 (m, 1H), 7.13–7.09 (m, 1H), 6.47 (s, 1H), 4.00 (s, 2H), 3.50 (s, 3H), 3.10 (t, J_1 = 6.0 Hz, J_2 = 12.0 Hz, 2H), 2.51 (t, J_1 = 6.0 Hz, J_2 = 6.0 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.53, 152.36, 145.50, 142.01, 136.76, 126.32, 125.84, 122.46, 120.60, 119.26, 116.41, 112.70, 110.78, 106.98, 42.24, 30.22, 24.47; HRMS ((+)-ESI): *m/z* = 280.1429 (calcd. 280.1444 for $C_{17}H_{17}N_3O$, $[M + H]^+$).

*4-(7-Chloroquinolin-4-yl)-1-methyl-5,6,7,8-tetrahydro-1,6-naphthyridin-2(1*H*)-one hydrochloride* (***10b***). White solid; yield 83%; ^{1}H NMR (600 MHz, MeOD): δ 7.80 (d, *J* = 6.0 Hz, 1H), 6.83 (d, *J* = 6.0 Hz, 1H), 6.52–6.48 (m, 2H), 6.42 (dd, J_1 = 12.0 Hz, J_2 = 6.0 Hz, 1H), 5.04 (s, 1H), 2.12 (s, 3H), 2.02–1.98 (m, 2H), 1.72–1.70 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.89, 153.00, 145.89, 145.02, 143.39, 141.68, 138.68, 132.04, 128.38, 125.69, 122.22, 120.30, 117.87, 106.05, 41.40, 40.00, 30.29, 24.09; HRMS ((+)-ESI): *m/z* = 348.0869 (calcd. 348.0874 for $C_{18}H_{16}ClN_3O$, $[M + Na]^+$).

*4-(2-Methoxypyridin-3-yl)-1-methyl-5,6,7,8-tetrahydro-1,6-naphthyridin-2(1*H*)-one hydrochloride* (***10c***). Brown solid; yield 59%; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.21 (dd, J_1 = 6.0 Hz, J_2 = 6.0 Hz, 1H), 7.39 (dd, J_1 = 12.0 Hz, J_2 = 6.0 Hz, 1H), 6.96 (dd, J_1 = 12.0 Hz, J_2 = 6.0 Hz, 1H), 6.37 (s, 1H), 4.75 (s, 2H), 3.93 (s, 3H), 3.54 (s, 3H), 3.18 (s, 2H), 2.72 (t, J_1 = 6.0 Hz, J_2 = 12.0 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.64, 160.25, 147.75, 147.34, 141.64, 138.40, 120.95, 118.04, 116.83, 114.24, 53.62, 44.77, 43.00, 30.03, 28.02; HRMS ((+)-ESI): *m/z* = 272.1372 (calcd. 272.1394 for $C_{15}H_{17}N_3O_2$, $[M + H]^+$).

*4-(1,5-Dimethyl-1*H*-pyrazol-4-yl)-1-methyl-5,6,7,8-tetrahydro-1,6-naphthyridin-2(1*H*)-one hydrochloride* (***10d***). White solid; yield 64%; ^{1}H NMR (600 MHz, $CDCl_3$): δ 6.59 (s, 1H), 5.29 (s, 1H), 2.76 (s, 2H), 2.59 (s, 3H), 2.35 (s, 3H), 2.24 (t, J_1 = 12.0 Hz, J_2 = 6.0 Hz, 2H), 1.92–1.90 (m, 3H), 0.99 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.69, 152.38, 144.48, 143.24, 141.29, 125.00, 116.21, 115.09, 111.62, 99.99, 42.14, 40.04, 35.38, 31.26; HRMS ((+)-ESI): *m/z* = 259.1540 (calcd. 259.1553 for $C_{14}H_{18}N_4O$, $[M + H]^+$).

*Methyl-4-(pyridin-4-yl)-5,6,7,8-tetrahydro-1,6-naphthyridin-2(1*H*)-one hydrochloride* (***10e***). Yellow solid; yield 77%; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.17 (s, 1H), 7.92–7.91 (m, 1H), 7.37 (dd, J_1 = 12.0 Hz, J_2 = 6.0 Hz, 2H), 6.79 (s, 1H), 3.16 (s, 3H), 2.42 (t, J_1 = 12.0 Hz, J_2 = 6.0 Hz, 2H), 1.99–1.97 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 162.77, 158.72, 147.51, 129.17, 114.35, 112.69, 108.72, 93.83, 41.17, 39.54, 31.46, 25.67; HRMS ((+)-ESI): *m/z* = 242.1879 (calcd. 242.1895 for $C_{14}H_{16}N_3O$, $[M + H]^+$).

*Methyl-4-(2-(trifluoromethoxy)phenyl)-5,6,7,8-tetrahydro-1,6-naphthyridin-2(1*H*)-one hydrochloride* (***10f***). White solid; yield 69%; ^{1}H NMR (600 MHz, MeOD): δ 7.15–7.14 (m, 1H), 6.68 (t, J_1 = 12.0 Hz, J_2 = 6.0 Hz, 2H), 6.42–6.41 (m, 2H), 5.51 (s, 1H), 3.17–3.15 (m, 2H), 2.92 (s, 3H), 2.45 (t, J_1 = 12.0 Hz, J_2 = 12.0 Hz, 2H), 1.99 (d, *J* = 12.0 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.92, 153.17, 141.59, 136.81, 131.52, 131.19, 130.56, 128.79, 125.94, 124.44, 116.35, 109.11, 45.17, 40.12, 30.83, 24.05; HRMS ((+)-ESI):

m/z = 325.1221 (calcd. 325.1237 for $C_{16}H_{16}F_3N_2O_2$, $[M + H]^+$).

*6-(1-Methyl-2-oxo-1,2,5,6,7,8-hexahydro-1,6-naphthyridin-4-yl) nicotinonitrile hydrochloride (**10g**).* White solid; yield 85%; ^{1}H NMR (600 MHz, MeOD): δ 8.93 (s, 1H), 8.26 (dd, J_1 = 12.0 Hz, J_2 = 6.0 Hz, 1H), 7.80 (d, J = 12.0 Hz, 1H), 6.71 (s, 1H), 3.59 (s, 3H), 3.22–3.21 (m, 3H), 3.17 (t, J_1 = 12.0 Hz, J_2 = 6.0 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 158.00, 151.52, 142.72, 141.34, 136.82, 136.54, 123.97, 117.46, 115.84, 110.10, 107.74, 42.43, 39.96, 30.42, 24.18; HRMS ((+)-ESI): *m/z* = 267.1225 (calcd. 267.1240 for $C_{15}H_{14}N_4O$, $[M + H]^+$).

*Methyl-4-(5-methylpyridin-3-yl)-5,6,7,8-tetrahydro-1,6-naphthyridin-2(1*H*)-one hydrochloride (**10h**).* Light yellow solid; yield 73%;^{1}H NMR (600 MHz, $CDCl_3$): δ 7.58 (d, J_1 = 6.0 Hz, 2H), 7.28 (s, 1H), 5.30 (s, 1H), 2.77 (s, 2H), 2.36 (s, 3H), 2.03–2.01 (m, 2H), 1.96 (t, J_1 = 12.0 Hz, J_2 = 6.0 Hz, 2H), 1.37 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 146.54, 144.99, 142.63, 141.69, 139.52, 138.00, 135.45, 134.43, 118.83, 105.94, 41.79, 40.15, 29.96, 24.02, 17.12; HRMS ((+)-ESI): *m/z* = 256.1429 (calcd. 256.1444 for $C_{15}H_{17}N_3O$, $[M + H]^+$).

*4-(4-Ethoxyphenyl)-1-methyl-5,6,7,8-tetrahydro-1,6-naphthyridin-2(1*H*)-one hydrochloride (**10i**).* White solid; yield 81%; ^{1}H NMR (600 MHz, MeOD): δ 5.92 (d, J = 12.0 Hz, 2H), 5.65 (d, J = 12.0 Hz, 2H), 5.27 (s, 1H), 2.74–2.66 (m, 4H), 2.35 (s, 3H), 2.27–2.16 (m, 3H), 1.89 (s, 5H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 161.91, 160.38, 154.82, 142.79, 129.28, 127.33, 114.72, 111.57, 66.74, 63.43, 42.64, 40.16, 31.36, 24.18, 13.65; HRMS ((+)-ESI): *m/z* = 285.1589 (calcd. 285.1598 for $C_{17}H_{20}N_2O_2$, $[M + H]^+$.

Biological evaluation. Cytotoxicity *in vitro* against tumor cell lines (HepG2 and MOLM-13) of them was also evaluated by using the CCK8 method [27].

Cytotoxicity. Cytotoxicity was evaluated by the Cell Counting Kit-8 (CCK8, DOJINDO, Japan) assay. The cells were seeded at 5000 cells per well into 96-well microplate in 100 μL of growth medium. Cells were incubated at 37°C and 5% CO_2 overnight. In the next day, 100 μL per well of diluted inhibitor in growth medium was added with the final concentration from 32 nM to 500 μM. The cells were treated with DMSO control. A series of dilutions are made in 0.1% DMSO in assay medium so that the final concentration of DMSO is 0.1% in all of treatments. Cells were incubated at 37°C and 5% CO_2 for 72 h. Then the CCK8 was added at 10 μL per well. The plates were incubated at 37°C for 2 h, and after that, the plates were recorded by measuring absorbance at 450 nm with the reference wavelength of 630 nm using an EnVision Multilabel Reader (PerkinElmer).

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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