3-(吡啶-3-基)-4-(吡唑并[3,4-*c*]哒嗪-3-基)马来酰亚胺脱氢类 异柠檬酸酶-1 突变体高效抑制剂的合成与评价

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摘要 合成了一系列新型的3-(吡啶-3-基)-4-(吡唑并[3,4-*c*]哒嗪-3-基)马来酰亚胺,并评价了其对于异柠檬酸脱氢酶1突变体(R132H)的抑制活性。大多数化合物对 IDH1-R132H 表现出较强的活性。其中化合物 3-(1-(3-(1-(1*H*-咪唑-1-基)丙基)-6-溴-1*H*-吡啶-3-基)-4-(1-甲基-1*H*-吡唑并[3,4-*c*]哒嗪-3-基)-1*H*-吡咯-2,5-二酮(**9b**)是最有前途的 IDH1-R132H 抑制剂, IC₅₀ 值为 31 nmol/L,并能显著抑制异柠檬酸脱氢酶 1 突变(R132H)型人脑星形胶质母细胞瘤细胞中 2-HG 的产生。根据实验数据进行了初步的构效关系讨论和分子模拟研究。

关键词 3-(吡啶-3-基)-4-(吡唑并[3,4-*c*]哒嗪-3-基)马来酰亚胺; 异柠檬酸脱氢酶 1 突变体(R132H)抑制剂; 合成; 生物活性

Synthesis and Evaluation of 3-(Indol-3-yl)-4-(pyrazolo[3,4-*c*]pyridazin-3-yl)maleimides as Potent Mutant Isocitrate Dehydrogenase-1 Inhibitors

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Abstract A series of novel 3-(indol-3-yl)-4-(pyrazolo[3,4-*c*]pyridazin-3-yl)maleimides were synthesized and evaluated for their inhibitory activity against mutant isocitrate dehydrogenase-1 (R132H). Most compounds exhibited potent potency to IDH1-R132H. Among them, compound **9b** was the most promising IDH1-R132H inhibitor with IC₅₀ values of 31 nmol/L and could significantly inhibit the production of 2-HG in human brain astroblastoma cells with mutant isocitrate dehydrogenase-1 (R132H). Preliminary structure-activity relationship and molecular modeling studies were discussed based on the experimental data obtained.

Keywords 3-(indol-3-yl)-4-(pyrazolo[3,4-*c*]pyridazin-3-yl)maleimide; mutant isocitrate dehydrogenase-1 (R132H) inhibitor; synthesis; biological activity

1 Introduction

Isocitrate dehydrogenase-1 (IDH1) is a key rate-limiting

enzyme in tricarboxylic acid cycle (TCA). Studies have shown that IDH1 has a high mutation rate in several tumors,^[1-2] including acute myeloid leukemia (AML),^[3-6]

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acute lymphoblastic leukemia (ALL),^[7-8] myelodysplastic syndrome (MDS),^[9-10] prostate cancer,^[11] glioma,^[12] chondrosarcoma^[13-14] and cholangiocarcinoma.^[15] The mutations of IDH1 mainly include R132H, R132C, R132L, R132S, R132G and R132Q, among which R132H is the main mutation and accounts for about 90% of all cases. After mutation, the functions of IDH1 change in two ways. On one hand, the mutation destroys the affinity of the enzyme, resulting in a decrease in the ability of the enzyme to bind to isocitrate (ICT) (K_i and K_m values increase by about 1000 times), and a significant decrease of α -KG, which is the decarboxylation product of isocitric acid catalyzed by IDH1. On the other hand, the mutation produces a new catalytic function, which can reduce α -KG to 2-hydroxyglutarate (2-HG), and therefore lead to the accumulation of 2-HG in mutant tumor cells (Scheme 1). Since 2-HG and α -KG share similar structures, they compete with each other in binding to some enzymes. Both of them stated aspects result in a decrease in the activity of some α -KG dependent dioxygenases, leading to the oncogenesis and development of tumors.^[16-17] Therefore, IDH1-R132H has become a potentially attractive anti-tumor target, and screening of appropriate inhibitors has become a hot spot in the development of new anti-tumor drugs.

Scheme 1 Reactions catalyzed by mutant isocitrate dehydrogenase-1

In recent years, the development of drugs targeting IDH1 mutants has been widely studied, and inhibitors with several different structure types have been reported. Among them, **AG-120**, which is developed by Agios Pharmaceuticals and used in the treatment of acute myeloid leukemia (AML), has been approved on July 20th, 2018.^[18] In addition, **IDH-305** that developed by Novartis Pharmaceuticals and **BAY-1436032** that developed by Bayer AG have been used for clinical researches.^[19-20]

Figure 1 IDH1-R132H inhibitors

In our previous studies we have developed two novel classes of 3-(7-azaindoyl)-4-indolylmaleimide and 3-(7-azaindazolyl)-4-indolylmaleimide IDH1-R132H inhibitors

(compound **A**, $IC_{50} = 280 \pm 14$ nmol/L; compound **B**, $IC_{50} = 61 \pm 5.2$ nmol/L) (Figure 1).^[21-22] Molecular docking studies showed that the substitution of the C(6) in the 7-azadiazole ring by a N resulted in the ability to form a stable hydrogen bond with the amino group of Arg119 using the N atom as a hydrogen bond acceptor, which may improve the inhibitory activity of the compound. For the reasons stated above, in this study, a pyrazolido[3,4-*c*]pyridazine ring was used to replace the 7-azadiazole ring of 3-(7-azaindazolyl)-4-indolylmaleimides. The substituents on the indole ring and the N atoms in maleimide, indole, and 7-azaindazole rings were also modified to study the structure-activity relationship. Thus, a series of 3-(indol-3-yl)-4-(pyrazolo[3,4-*c*]pyridazin-3-yl)maleimide small-molecule inhibitors of IDH1-R132H were designed and synthesized and the biological activity was studied. The protein binding patterns of these compounds were also studied.

2 Results and discussion

2.1 Chemistry

The synthetic route of compounds **9a~9i** is illustrated in Scheme 2. Diazotizing **1** with hydrochloric acid and $NaNO_2$, then reacted with KI to afford **2**. **2** was treated with $EtMgBr$, then reacted with $Sn(Bu)_3Cl$ in dry THF to result in **3**.^[23] Alkylation of **3** with methyl iodide, bromoethane or 1-bromobutane in the presence of NaH in dry DMF afforded **4a~4c** respectively. **5a~5e** were treated with $EtMgBr$, then reacted with 3,4-dichloro-1-phenyl-1*H*-pyrrole-2,5-dione in dry THF to result in **6a~6e**. Stille coupling of **4a~4c** with **6a~6e** using $Pd(Ph_3)_2Cl_2$ as a catalyst in dry toluene afforded intermediate **7a~7g**.^[24] Alkylation of **7a~7g** with 1-(3-chloropropyl)-1*H*-imidazole in the presence of NaH in dry DMF yielded **8a~8g**. Target compounds **9a~9g** were synthesized by treating **8a~8g** with melted NH_4OAc . Treatment of **7b** with melted NH_4OAc afforded **9h**. Reaction of **8b** with methylamine in alcohol afforded compound **9i**.

To efficiently synthesize the target compounds, a preliminary study of the stille coupling reaction was conducted. Compounds **4a** and **6a** were used as model substrates to investigate the reaction conditions, including factors such as palladium catalyst, additive, solvent type and reaction temperature (Table 1). Product **7a** was initially obtained with a yield of 76.9% via the reaction between **4a** (0.57 mmol) and **6a** (0.62 mmol) with $Pd(PPh_3)_2Cl_2$ (15 mol%) as the catalyst, LiCl (1.30 mmol) as the additive, and anhydrous PhMe (25 mL) as the solvent, performed at 100 °C for 2 h under a nitrogen atmosphere. Further studies showed that replacing the catalyst with $Pd(PPh_3)_4$ or $PdCl_2(dppf)$, or replacing the additive LiCl with CsF caused a decrease in the yield. When 1,4-dioxane was used as the solvent, the yield was significantly increased (94.5%) and reducing the amount of catalyst or lowering the temperature led to a significant decrease in yield. Therefore, the optimal conditions were a reaction temperature of 100 °C with 1,4-dioxane as the solvent, $Pd(PPh_3)_2Cl_2$ as the catalyst, and LiCl

Reagents and conditions: (a) HCl, NaNO₂, KI; (b) EtMgBr, Sn(Bu)₃Cl, THF; (c) NaH, R¹X; (d) EtMgBr, 3,4-dichloro-1-phenyl-1*H*-pyrrole-2,5-dione, THF; (e) Pd(PPh₃)₂Cl₂, LiCl, toluene; (f) 1-(3-chloropropyl)-1*H*-imidazole, NaH, DMF; (g) NH₄OAc; (h) NH₄OAc; (i) CH₃NH₂

Scheme 2 Synthetic route of compounds **9a**~**9i**

Table 1 Optimization of the stille coupling reaction conditions^a

Entry	Pd-cat	Solvent	Temp./°C	Pd-cat/mol%	Additive	Yield ^b /%
1 ^c	Pd(PPh ₃) ₂ Cl ₂	PhMe	100	15	LiCl	76.9
2	Pd(PPh ₃) ₄	PhMe	100	15	LiCl	69.9
3	PdCl ₂ (dppf)	PhMe	100	15	LiCl	56.2
4	Pd(PPh ₃) ₂ Cl ₂	PhMe	100	15	CsF	68.3
5	Pd(PPh ₃) ₂ Cl ₂	PhMe	100	15	—	39.3
6	Pd(PPh ₃) ₂ Cl ₂	DMF	100	15	LiCl	22.9
7	Pd(PPh ₃) ₂ Cl ₂	1,4-Dioxane	100	15	LiCl	94.5
8	Pd(PPh ₃) ₂ Cl ₂	CH ₃ CN	100	15	LiCl	Trace
9	Pd(PPh ₃) ₂ Cl ₂	1,4-Dioxane	100	10	LiCl	77.6
10	Pd(PPh ₃) ₂ Cl ₂	1,4-Dioxane	100	5	LiCl	61.5
11	Pd(PPh ₃) ₂ Cl ₂	1,4-Dioxane	90	15	LiCl	63.7

^a Reaction conditions: **4a** (0.57 mmol), **6a** (0.62 mmol), Pd-cat, solvent (0.023 mol/L), N₂ atmosphere, 2 h. PhMe: methylbenzene; DMF: dimethylformamide; MeCN: acetonitrile. ^b Isolated yields.

as the additive.

2.2 Enzymatic activity

The IDH1-R132H inhibitory potency of all target compounds was tested. In addition, the assay of inhibitory activity toward IDH1/WT was also conducted to determine the selectivity of the selected compounds. **AG-120** was used as a reference compound in these assays. The results are listed in Tables 2 and 3.

Table 2 IDH1-R132H inhibitory activity of the target compounds **8b** and **9a~9i**

Compd.	R ¹	R ²	R ³	R ⁴	IC ₅₀ ± SE ^a / (nmol·L ⁻¹)
9a	H	(CH ₂) ₃ N	Me	H	148 ± 46
9b	6-Br	(CH ₂) ₃ N	Me	H	31 ± 2
9c	6-F	(CH ₂) ₃ N	Me	H	127 ± 53
9d	5-F	(CH ₂) ₃ N	Me	H	84 ± 31
9e	6-Me	(CH ₂) ₃ N	Me	H	42 ± 12
9f	6-Br	(CH ₂) ₃ N	Et	H	37 ± 3
9g	6-Br	(CH ₂) ₃ N	<i>n</i> -Bu	H	76 ± 3
9h	6-Br	H	Me	H	1495 ± 258
9i	6-Br	(CH ₂) ₃ N	Me	Me	351 ± 23
8b	6-Br	(CH ₂) ₃ N	Me	Ph	≥ 10000
AG-120					199 ± 81

^a SE: standard error mean.

Table 3 IDH1/WT inhibitory activity of selected compounds

Compd.	IC ₅₀ / (μmol·L ⁻¹)
9b	≥ 10
9d	≥ 10
9e	≥ 10
9f	≥ 10
9g	≥ 10

As shown in Table 2, most of the tested compound showed similar or more potent IDH1-R132H inhibitory activity in comparison with **AG-120**. Structure-activity relationship (SAR) analysis showed that introduction of a small hydrophobic group such as bromine (**9b**, IC₅₀ = 31 ± 2 nmol/L) and methyl (**9e**, IC₅₀ = 42 ± 12 nmol/L) at the 6-position of indole ring led to about four-fold increase of the activity as compared to **9a** (IC₅₀ = 148 ± 46 nmol/L). Fluorine at 5- or 6-position of the indole ring was tolerated for the activity.

Introduction of a 3-(1*H*-imidazol-1-yl)propyl on the indole nitrogen (**9b**, IC₅₀ = 31 ± 2 nmol/L), which may form hydrogen bond with the protein, resulted in about 50-fold enhancement of the activity as compared to **9h** (IC₅₀ = 1495 ± 258 nmol/L). A small hydrophobic methyl (**9b**) or ethyl (**9f**, IC₅₀ = 37 ± 3 nmol/L) on N¹-position of the pyrazolo[3,4-*c*]pyridazine ring was tolerated for the activity. However, replacement of methyl or ethyl with a larger butyl (**9g**, IC₅₀ = 76 ± 3 nmol/L) led to some loss in IDH1-R132H potency. Comparison of the inhibitory activity of **9b** with **8b** (IC₅₀ ≥ 10000 nmol/L) and **9i** (IC₅₀ = 351 ± 23 nmol/L) revealed that the NH of the imide moiety is important for the activity. The data in Table 3 revealed that all the selected compounds **9b**, **9d**, **9e**, **9f** and **9g** showed high selectivity on IDH1/WT.

2.3 Cellular activity

IDH1 mutants catalyze the conversion of α-KG to 2-HG. Currently, 2-HG is used as a biomarker for IDH1 mutants.^[25-27] Therefore, the down-regulation of intracellular 2-HG by a compound is the most direct indicator of the inhibition of IDH1 mutants. Using **AG-120** as a reference, the ability of compounds **9b**, **9f** and **9g** to reduce 2-HG production in U87MG cells was investigated using a LC-MS/MS method. As shown in Figure 2, at a concentration of 10 μmol/L, compounds **9b**, **9f** and **9g** effectively inhibited the production of 2-HG in U87MG cells expressing IDH1-R132H. Compound **9b** had the highest activity with an inhibition rate of 87%. In addition, the activity of the compound was dependent on the concentration. When the

Figure 2 Selected compounds inhibited 2-HG production in IDH1-R132H expressing U87MG cells

concentration was reduced to 1 $\mu\text{mol/L}$, the activity was decreased significantly. Unfortunately, although compounds **9b**, **9f** and **9g** had better inhibitory activity toward IDH1-R132H than the positive control **AG-120**, the ability of the compounds to inhibit 2-HG production in cells was significantly lower than that of **AG-120**. This relatively low cellular activity may be because of an insufficient ability of the designed compounds to penetrate cells.

2.4 Molecular modeling study

To investigate the possible binding patterns of compound **9b** to IDH1-R132H, molecular docking was performed using the reported IDH1-R132H structure (PDB ID: 5DE1) by using the CDOCKER module (Discovery Studio, version 2.5; Accelrys, San Diego, CA, USA, 2008). Docking results showed that the CDOCKER ENERGY of compound **9b** (-75.8 kJ/mol) was lower than that of ligand (-56.9 kJ/mol) in protein 5DE1, indicating a stronger binding affinity. Then we outputted the results using pymol 1.8. Figure 3 clearly shows that the NH and carbonyl groups on the maleimide ring of **9b** can form important interactions with the hinge region residues Ile128 and Ala111 of IDH1-R132H via a hydrogen bond network. The nitrogen atom at the 6th and 7th positions in the pyrazolo[3,4-*c*]pyridazine moiety of compound **9b** acts as a hydrogen bond acceptor to form two other hydrogen bonds with Arg119. And the methyl group was inserted into a smaller hydrophobic pocket consisting of Arg119 and Trp124. The indole moiety of compound **9b** has a π - π interaction (T-shaped) with the Trp124 residue. In addition, the 6-position bromine atom on the indole ring can be inserted into a hydrophobic pocket composed of Val255 and Met259 which is similar to that reported by Ref. [28]. Finally, the 3-position nitrogen of the imidazole ring is closer to the NH of Arg119 and Leu120, which makes it possible for the N atom to interact with Arg119 and Leu120 via a water bridge.

Figure 3 Docking of **9b** to IDH1-R132H crystal structure

3 Conclusions

Ten novel 3-(indol-3-yl)-4-(pyrazolo[3,4-*c*]pyridazin-3-yl)maleimide IDH1-R132H inhibitors were designed and synthesized. Compounds **9b**, **9d**, **9e**, **9f** and **9g** showed strong inhibitory activity to IDH1-R132H and good selectivity to wild-type IDH1. Compound **9b** could effectively inhibit the production of 2-HG in U87MG cells expressing IDH1-R132H. Studies of the structure-activity relationship and the protein binding patterns provided theoretical guid-

ance for further researches.

4 Experimental section

4.1 Instruments and reagents

All reagents used in the synthesis were obtained commercially and used without further purification, unless otherwise specified. Melting points were determined with a BüCHI Melting Point B-450 apparatus (Büchi Labortechnik, Flawil, Switzerland) and are uncorrected. ^1H NMR spectra were recorded using TMS as the internal standard at 500 MHz and the coupling constants are reported in Hz. The reactions were followed by thin-layer chromatography (TLC) on glass-packed precoated silica gel plates and visualized in an iodine chamber or with a UV lamp. Tetrahydrofuran (THF) was distilled from sodium-benzophenone. DMF was distilled from calcium hydride. Preparation methods and physicochemical properties for compounds **6a**~**6e** were reported in Ref. [22].

4.2 Synthesis

4.2.1 Synthesis of **2**

To a mixture of 1*H*-pyrazolo[3,4-*c*]pyridazin-3-amine (**1**, 11.0 g, 81 mmol), concentrated hydrochloric acid (68 mL) and water (40 mL), a solution of NaNO_2 (6.2 g, 90 mmol) in water (90 mL) was added dropwise at $0\sim 5^\circ\text{C}$. After addition, the mixture was stirred for additional 30 min at that temperature. After that, appropriate amount of urea was added to destroy the excess NaNO_2 . The resulted mixture was added in batches to a solution of KI (46.0 g, 280 mmol) in water (300 mL) at room temperature and then stirred for another 8 h. Then the mixture was adjusted to weakly alkaline with saturated NaHCO_3 solution, filtered and washed with water. The filter cake was dried in vacuum to afford 3-iodo-1*H*-pyrazolo[3,4-*c*]pyridazine (**2**) (12.0 g, 60.0%) as a brown solid, m.p. $238\sim 240^\circ\text{C}$. ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ : 14.91 (s, 1H), 9.18 (d, $J=5.5$ Hz, 1H), 7.91 (d, $J=5.6$ Hz, 1H); ESI-MS m/z : 247 $[\text{M}+\text{H}]^+$.

4.2.2 Synthesis of **3**

To a solution of **2** (6.0 g, 24 mmol) in dry tetrahydrofuran (THF) (500 mL), EtMgBr (48 mmol) in dry Et_2O (48 mL) was added dropwise at $-5\sim 0^\circ\text{C}$. After stirring for 30 min, a solution of $\text{Sn}(\text{Bu})_3\text{Cl}$ (19.5 g, 60 mmol) in dry THF (15 mL) was added dropwise to the mixture at $-5\sim 0^\circ\text{C}$. The reaction mixture was stirred at $-5\sim 0^\circ\text{C}$ for 1 h, then at room temperature for 1 h. The mixture was poured into NH_4Cl saturation solution (500 mL), and extracted with EtOAc (300 mL $\times$ 3). The combined organic layers were washed with brine, dried over Na_2SO_4 , concentrated in vacuum and purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate ($V:V=2:1$) to afford 3-(tributylstannyl)-1*H*-pyrazolo[3,4-*c*]pyridazine (**3**)^[22] (2.6 g, 38.6%) as a yellow liquid. ^1H NMR (500 MHz, CDCl_3) δ : 12.62 (s, 1H), 9.12 (d, $J=5.5$ Hz, 1H), 7.86 (d, $J=5.5$ Hz, 1H), 1.72~1.52 (m, 6H), 1.38~1.27 (m, 12H), 0.90 (t, $J=7.3$ Hz, 9H); MS (ESI) m/z : 411 $[\text{M}+\text{H}]^+$. Anal. calcd for $\text{C}_{17}\text{H}_{30}\text{N}_4\text{Sn}$: C 49.90, H 7.39, N 13.69; found C

49.76, H 7.63, N 13.57.

4.2.3 Synthesis of **4**

To a solution of **3** (2.2 g, 5.3 mmol) in dry *N,N*-dimethylformamide (DMF) (40 mL), 60% NaH (0.24 g, 6.0 mmol) was added in batches at 0~5 °C, and the mixture was then stirred for another 0.5 h at that temperature. After that, CH₃I (0.91 g, 6.4 mmol) was added and stirred at room temperature for 1 h. The resulted mixture was poured into water (300 mL), extracted with dichloromethane (DCM) (100 mL × 3) and the combined organic layers were washed with brine, dried over Na₂SO₄, concentrated in vacuum and purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate (*V*: *V*=4:1) to afford 1-methyl-3-(tributylstannyl)-1*H*-pyrazolo[3,4-*c*]pyridazine (**4a**) (1.5 g, 67.5%) as a yellow liquid. ¹H NMR (500 MHz, CDCl₃) δ: 9.05 (d, *J*=5.5 Hz, 1H), 7.76 (d, *J*=5.5 Hz, 1H), 4.39 (s, 3H), 1.64~1.56 (m, 6H), 1.42~1.31 (m, 12H), 0.89 (t, *J*=7.3 Hz, 9H); MS (ESI) *m/z*: 425 [M+H]⁺. Anal. calcd for C₁₈H₃₂N₄Sn: C 51.09, H 7.62, N 13.24; found C 51.17, H 7.72, N 13.08.

1-Ethyl-3-(tributylstannyl)-1*H*-pyrazolo[3,4-*c*]pyridazine (**4b**): Reaction of **3** with bromoethane afforded **4b** in 45.8% yield as a yellow liquid. ¹H NMR (500 MHz, CDCl₃) δ: 9.04 (d, *J*=5.5 Hz, 1H), 7.75 (d, *J*=5.5 Hz, 1H), 4.84 (q, *J*=7.2 Hz, 2H), 1.65~1.60 (m, 6H), 1.56 (t, *J*=7.2 Hz, 3H), 1.40~1.23 (m, 12H), 0.89 (t, *J*=7.3 Hz, 9H); MS (ESI) *m/z*: 439 [M+H]⁺. Anal. calcd for C₁₉H₃₄N₄Sn: C 52.20, H 7.84, N 12.81; found C 52.33, H 7.75, N 12.66.

1-Butyl-3-(tributylstannyl)-1*H*-pyrazolo[3,4-*c*]pyridazine (**4c**): Reaction of **3** with bromobutane afforded **4c** in 52.4% yield as a yellow liquid. ¹H NMR (500 MHz, CDCl₃) δ: 9.04 (d, *J*=5.5 Hz, 1H), 7.76 (d, *J*=5.6 Hz, 1H), 4.79 (t, *J*=7.2 Hz, 2H), 2.08~1.98 (m, 2H), 1.70~1.50 (m, 6H), 1.43~1.18 (m, 14H), 0.97 (t, *J*=7.4 Hz, 3H), 0.89 (t, *J*=7.3 Hz, 9H); MS (ESI) *m/z*: 467 [M+H]⁺. Anal. calcd for C₂₁H₃₈N₄Sn: C 54.21, H 8.23, N 12.04; found C 54.40, H 8.15, N 12.22.

4.2.4 Synthesis of **7**

Under a nitrogen atmosphere, a mixture of **4a** (240 mg, 0.57 mmol), **6a** (201 mg, 0.62 mmol), Pd(Ph₃)₂Cl₂ (60 mg, 0.085 mmol) and LiCl (55 mg, 1.30 mmol) in dry 1,4-dioxane (25 mL) was stirred at 100 °C for 2 h. After cooling, the mixture was poured into water (50 mL), and then extracted with EtOAc (50 mL × 3). The combined organic layer was washed with brine, dried over Na₂SO₄, concentrated in vacuum and purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate (*V*: *V*=4:1) to give 3-(1*H*-indol-3-yl)-4-(1-methyl-1*H*-pyrazolo[3,4-*c*]pyridazin-3-yl)-1-phenyl-1*H*-pyrrole-2,5-dione (**7a**) (202 mg, 94.5%) as a red solid. m.p. 238~240 °C; ¹H NMR (500 MHz, CDCl₃) δ: 12.13 (s, 1H), 9.15 (d, *J*=5.7 Hz, 1H), 8.30 (d, *J*=3.0 Hz, 1H), 8.05 (d, *J*=5.7 Hz, 1H), 7.60~7.52 (m, 5H), 7.50~7.41 (m, 2H), 6.81~6.73 (m, 1H), 6.37 (d, *J*=8.0 Hz, 1H), 4.21 (s, 3H); MS (ESI) *m/z*: 421 [M+H]⁺. Anal. calcd for C₂₄H₁₆N₆O₂: C 68.56, H 3.84, N 19.99; found C 68.43, H 3.61, N 20.09.

3-(6-Bromo-1*H*-indol-3-yl)-4-(1-methyl-1*H*-pyrazolo[3,4-*c*]pyridazin-3-yl)-1-phenyl-1*H*-pyrrole-2,5-dione (**7b**): Reaction of **4a** with **6b** afforded **7b** in 44.6% yield as a red solid. m.p. 122~124 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ: 12.17 (s, 1H), 9.18 (d, *J*=5.7 Hz, 1H), 8.31 (d, *J*=2.1 Hz, 1H), 8.08 (d, *J*=5.6 Hz, 1H), 7.67 (d, *J*=1.8 Hz, 1H), 7.60~7.52 (m, 4H), 7.50~7.42 (m, 1H), 6.98 (dd, *J*=8.6, 1.9 Hz, 1H), 6.47 (d, *J*=8.6 Hz, 1H), 4.24 (s, 3H); MS (ESI) *m/z*: 499 [M+H]⁺. Anal. calcd for C₂₄H₁₅BrN₆O₂: C 57.73, H 3.03, N 16.83; found C 57.88, H 3.15, N 16.96.

3-(6-Fluoro-1*H*-indol-3-yl)-4-(1-methyl-1*H*-pyrazolo[3,4-*c*]pyridazin-3-yl)-1-phenyl-1*H*-pyrrole-2,5-dione (**7c**): Reaction of **4a** with **6c** afforded **7c** in 72.7% yield as a red solid. m.p. >250 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ: 12.13 (s, 1H), 9.18 (d, *J*=5.6 Hz, 1H), 8.30 (d, *J*=2.9 Hz, 1H), 8.09 (d, *J*=5.6 Hz, 1H), 7.59~7.53 (m, 4H), 7.50~7.42 (m, 1H), 7.27 (dd, *J*=9.6, 2.5 Hz, 1H), 6.70 (dd, *J*=9.3, 2.4 Hz, 1H), 6.49~6.43 (m, 1H), 4.23 (s, 3H); MS (ESI) *m/z*: 439 [M+H]⁺. Anal. calcd for C₂₄H₁₅FN₆O₂: C 65.75, H 3.45, N 19.17; found C 65.90, H 3.52, N 19.02.

3-(5-Fluoro-1*H*-indol-3-yl)-4-(1-methyl-1*H*-pyrazolo[3,4-*c*]pyridazin-3-yl)-1-phenyl-1*H*-pyrrole-2,5-dione (**7d**): Reaction of **4a** with **6d** afforded **7d** in 58.4% yield as a red solid. m.p. >250 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ: 12.19 (s, 1H), 9.20 (d, *J*=5.7 Hz, 1H), 8.37 (d, *J*=3.1 Hz, 1H), 8.10 (d, *J*=5.6 Hz, 1H), 7.59~7.53 (m, 4H), 7.52~7.42 (m, 2H), 6.96~7.00 (m, 1H), 6.24 (dd, *J*=10.7, 2.5 Hz, 1H), 4.26 (s, 3H); MS (ESI) *m/z*: 439 [M+H]⁺. Anal. calcd for C₂₄H₁₅FN₆O₂: C 65.75, H 3.45, N 19.17; found C 65.82, H 3.61, N 19.23.

3-(6-Methyl-1*H*-indol-3-yl)-4-(1-methyl-1*H*-pyrazolo[3,4-*c*]pyridazin-3-yl)-1-phenyl-1*H*-pyrrole-2,5-dione (**7e**): Reaction of **4a** with **6e** afforded **7e** in 67.6% yield as a red solid. m.p. >250 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ: 12.00 (s, 1H), 9.15 (d, *J*=5.6 Hz, 1H), 8.25 (s, 1H), 8.03 (d, *J*=5.6 Hz, 1H), 7.60~7.52 (m, 4H), 7.48~7.43 (m, 1H), 7.26 (s, 1H), 6.60 (dd, *J*=8.4, 1.5 Hz, 1H), 6.22 (d, *J*=8.2 Hz, 1H), 4.24 (s, 3H), 2.32 (s, 3H); MS (ESI) *m/z*: 435 [M+H]⁺. Anal. calcd for C₂₅H₁₈N₆O₂: C 69.11, H 4.18, N 19.34; found C 68.96, H 4.33, N 19.51.

3-(6-Bromo-1*H*-indol-3-yl)-4-(1-ethyl-1*H*-pyrazolo[3,4-*c*]pyridazin-3-yl)-1-phenyl-1*H*-pyrrole-2,5-dione (**7f**): Reaction of **4b** with **6b** afforded **7f** in 50.4% yield as a red solid. m.p. 152~153 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ: 12.17 (s, 1H), 9.22 (d, *J*=5.6 Hz, 1H), 8.30 (d, *J*=3.0 Hz, 1H), 8.19 (d, *J*=5.7 Hz, 1H), 7.68 (d, *J*=1.9 Hz, 1H), 7.58~7.55 (m, 4H), 7.49~7.42 (m, 1H), 6.95 (dd, *J*=8.6, 1.9 Hz, 1H), 6.44 (d, *J*=8.6 Hz, 1H), 4.67 (q, *J*=7.2 Hz, 2H), 1.33 (t, *J*=7.2 Hz, 3H); MS (ESI) *m/z*: 513 [M+H]⁺; Anal. calcd for C₂₅H₁₇BrN₆O₂: C 58.49, H 3.34, N 16.37; found C 58.63, H 3.21, N 16.49.

3-(6-Bromo-1*H*-indol-3-yl)-4-(1-butyl-1*H*-pyrazolo[3,4-*c*]pyridazin-3-yl)-1-phenyl-1*H*-pyrrole-2,5-dione (**7g**): Reaction of **4c** with **6b** afforded **7g** in 49.1% yield as a red solid. m.p. 220~221 °C; ¹H NMR (500 MHz, DMSO-*d*₆) δ: 12.16 (d, *J*=3.0 Hz, 1H), 9.22 (d, *J*=5.6 Hz, 1H), 8.26 (d, *J*=3.0 Hz, 1H), 8.19 (d, *J*=5.6 Hz, 1H), 7.68 (d, *J*=

1.8 Hz, 1H), 7.60~7.52 (m, 4H), 7.49~7.42 (m, 1H), 6.90 (dd, $J=8.5$, 1.9 Hz, 1H), 6.40 (d, $J=8.6$ Hz, 1H), 4.62 (t, $J=6.9$ Hz, 2H), 1.69~1.62 (m, 2H), 1.23~1.10 (m, 2H), 0.84 (t, $J=7.4$ Hz, 3H); MS (ESI) m/z : 541 $[M+H]^+$; Anal. calcd for $C_{27}H_{21}BrN_6O_2$: C 59.90, H 3.91, N 15.52; found C 59.99, H 4.01, N 15.43.

4.2.5 Synthesis of **8**

To a solution of **7a** (120 mg, 0.21 mmol) in dry DMF (30 mL), 60% NaH (13 mg, 0.32 mmol) was added at room temperature, and the mixture was stirred for 30 min. 1-(3-Chloropropyl)-1H-imidazole (61 mg, 0.42 mmol) was added and the mixture was stirred at 75 °C for 6 h. After cooling, the resulted mixture was poured into water (200 mL) and then extracted with EtOAc (100 mL $\times$ 3). The combined organic layers were washed with brine, dried over Na_2SO_4 , concentrated in vacuum and purified by flash column chromatography on silica gel using DCM/MeOH/Triethylamine (TEA) ($V:V:V=120:2:1$) to afford 3-(1-(3-(1H-imidazol-1-yl)propyl)-1H-indol-3-yl)-4-(1-methyl-1H-pyrazolo[3,4-c]pyridazin-3-yl)-1-phenyl-1H-pyrrole-2,5-dione (**8a**) (50 mg, 33.1%) as a red solid. m.p. 240~242 °C; 1H NMR (500 MHz, DMSO- d_6) δ : 9.16 (d, $J=5.6$ Hz, 1H), 8.36 (s, 1H), 8.08 (d, $J=5.6$ Hz, 1H), 7.71 (s, 1H), 7.58~7.55 (m, 4H), 7.52 (d, $J=8.2$ Hz, 1H), 7.48~7.43 (m, 1H), 7.27 (d, $J=1.4$ Hz, 1H), 7.17 (t, $J=7.8$ Hz, 1H), 6.96 (s, 1H), 6.86 (t, $J=7.6$ Hz, 1H), 6.54 (d, $J=8.1$ Hz, 1H), 4.32 (t, $J=7.3$ Hz, 2H), 4.22 (s, 3H), 4.07 (t, $J=7.1$ Hz, 2H), 2.36~2.30 (m, 2H); MS (ESI) m/z : 529 $[M+H]^+$. Anal. calcd for $C_{30}H_{24}N_8O_2$: C 68.17, H 4.58, N 21.20; found C 68.28, H 4.42, N 21.34.

3-(1-(3-(1H-imidazol-1-yl)propyl)-6-bromo-1H-indol-3-yl)-4-(1-methyl-1H-pyrazolo[3,4-c]pyridazin-3-yl)-1-phenyl-1H-pyrrole-2,5-dione (**8b**): Reaction of **7b** with 1-(3-chloropropyl)-1H-imidazole afforded **8b** in 38.0% yield as a red solid. m.p. 108~110 °C; 1H NMR (500 MHz, DMSO- d_6) δ : 9.22 (d, $J=5.6$ Hz, 1H), 8.33 (s, 1H), 8.20 (d, $J=5.7$ Hz, 1H), 7.86 (d, $J=1.8$ Hz, 1H), 7.84 (s, 1H), 7.61~7.53 (m, 4H), 7.50~7.42 (m, 1H), 7.31 (d, $J=1.5$ Hz, 1H), 7.04~7.01 (m, 2H), 6.55 (d, $J=8.6$ Hz, 1H), 4.33 (t, $J=7.4$ Hz, 2H), 4.24 (s, 3H), 4.09 (t, $J=7.2$ Hz, 2H), 2.36~2.31 (m, 2H); MS (ESI) m/z : 607 $[M+H]^+$. Anal. calcd for $C_{30}H_{23}BrN_8O_2$: C 59.32, H 3.82, N 18.45; found C 59.44, H 3.75, N 18.49.

3-(1-(3-(1H-imidazol-1-yl)propyl)-6-fluoro-1H-indol-3-yl)-4-(1-methyl-1H-pyrazolo[3,4-c]pyridazin-3-yl)-1-phenyl-1H-pyrrole-2,5-dione (**8c**): Reaction of **7c** with 1-(3-chloropropyl)-1H-imidazole afforded **8c** in 33.8% yield as a red solid. m.p. 199~201 °C; 1H NMR (500 MHz, DMSO- d_6) δ : 9.19 (d, $J=5.6$ Hz, 1H), 8.41 (s, 1H), 8.11 (d, $J=5.6$ Hz, 1H), 7.66 (s, 1H), 7.60~7.52 (m, 5H), 7.50~7.41 (m, 1H), 7.23 (s, 1H), 7.06 (td, $J=9.1$, 2.6 Hz, 1H), 6.93 (s, 1H), 6.44 (dd, $J=10.5$, 2.6 Hz, 1H), 4.30 (t, $J=7.3$ Hz, 2H), 4.25 (s, 3H), 4.03 (t, $J=7.2$ Hz, 2H), 2.34~2.28 (m, 2H); MS (ESI) m/z : 547 $[M+H]^+$; Anal. calcd for $C_{30}H_{23}FN_8O_2$: C 65.93, H 4.24, N 20.50; found C 66.06, H 4.49, N 20.38.

3-(1-(3-(1H-imidazol-1-yl)propyl)-5-fluoro-1H-indol-3-yl)-4-(1-methyl-1H-pyrazolo[3,4-c]pyridazin-3-yl)-1-phenyl-1H-pyrrole-2,5-dione (**8d**): Reaction of **7d** with 1-(3-chloropropyl)-1H-imidazole afforded **8d** in 39.3% yield as a red solid. m.p. 188~190 °C; 1H NMR (500 MHz, DMSO- d_6) δ : 9.19 (d, $J=5.6$ Hz, 1H), 8.41 (s, 1H), 8.11 (d, $J=5.6$ Hz, 1H), 7.66 (s, 1H), 7.56 (t, $J=3.7$ Hz, 5H), 7.43~7.47 (m, 1H), 7.23 (d, $J=1.3$ Hz, 1H), 7.04~7.08 (m, 2.6 Hz, 1H), 6.93 (t, $J=1.1$ Hz, 1H), 6.44 (dd, $J=10.5$, 2.6 Hz, 1H), 4.30 (t, $J=7.3$ Hz, 2H), 4.25 (s, 3H), 4.03 (t, $J=7.2$ Hz, 2H), 2.27~2.32 (m, 2H); MS (ESI) m/z : 547 $[M+H]^+$; Anal. calcd for $C_{30}H_{23}FN_8O_2$: C 65.93, H 4.24, N 20.50; found C 65.89, H 4.38, N 20.45.

3-(1-(3-(1H-imidazol-1-yl)propyl)-6-methyl-1H-indol-3-yl)-4-(1-methyl-1H-pyrazolo[3,4-c]pyridazin-3-yl)-1-phenyl-1H-pyrrole-2,5-dione (**8e**): Reaction of **7e** with 1-(3-chloropropyl)-1H-imidazole afforded **8e** in 42.2% yield as a red solid. m.p. 108~110 °C; 1H NMR (500 MHz, DMSO- d_6) δ : 9.16 (d, $J=5.7$ Hz, 1H), 8.30 (s, 1H), 8.06 (d, $J=5.6$ Hz, 1H), 7.91 (s, 1H), 7.58~7.54 (m, 4H), 7.51~7.41 (m, 1H), 7.37~7.32 (m, 1H), 7.30 (s, 1H), 7.06 (s, 1H), 6.68 (dd, $J=8.4$, 1.4 Hz, 1H), 6.37 (d, $J=8.2$ Hz, 1H), 4.29 (t, $J=7.3$ Hz, 2H), 4.24 (s, 3H), 4.10 (t, $J=7.1$ Hz, 2H), 2.38~2.26 (m, 5H); MS (ESI) m/z : 543 $[M+H]^+$. Anal. calcd for $C_{31}H_{26}N_8O_2$: C 68.62, H 4.83, N 20.65; found C 68.84, H 4.76, N 20.52.

3-(1-(3-(1H-imidazol-1-yl)propyl)-6-bromo-1H-indol-3-yl)-4-(1-ethyl-1H-pyrazolo[3,4-c]pyridazin-3-yl)-1-phenyl-1H-pyrrole-2,5-dione (**8f**): Reaction of **7f** with 1-(3-chloropropyl)-1H-imidazole afforded **8f** in 39.1% yield as a red solid. m.p. 116~118 °C; 1H NMR (500 MHz, DMSO- d_6) δ : 9.22 (d, $J=5.6$ Hz, 1H), 8.33 (s, 1H), 8.20 (d, $J=5.7$ Hz, 1H), 7.86 (d, $J=1.8$ Hz, 1H), 7.84 (s, 1H), 7.61~7.53 (m, 4H), 7.50~7.42 (m, 1H), 7.31 (d, $J=1.5$ Hz, 1H), 7.04~7.01 (m, 2H), 6.55 (d, $J=8.6$ Hz, 1H), 4.67 (q, $J=7.3$ Hz, 2H), 4.33 (t, $J=7.4$ Hz, 2H), 4.09 (t, $J=7.2$ Hz, 2H), 2.35~2.31 (m, 2H), 1.32 (t, $J=7.2$ Hz, 3H); MS (ESI) m/z : 621 $[M+H]^+$. Anal. calcd for $C_{31}H_{25}BrN_8O_2$: C 59.91, H 4.05, N 18.03; found C 59.76, H 4.15, N 18.22.

3-(1-(3-(1H-imidazol-1-yl)propyl)-6-bromo-1H-indol-3-yl)-4-(1-butyl-1H-pyrazolo[3,4-c]pyridazin-3-yl)-1-phenyl-1H-pyrrole-2,5-dione (**8g**): According to procedure used to prepare **8a**, the reaction of **7g** with 1-(3-chloropropyl)-1H-imidazole afforded **8g** in 38.2% yield as a red solid. m.p. 187~189 °C; 1H NMR (500 MHz, DMSO- d_6) δ : 9.22 (d, $J=5.7$ Hz, 1H), 8.28 (s, 1H), 8.21 (d, $J=5.7$ Hz, 1H), 7.86 (d, $J=1.8$ Hz, 1H), 7.68 (s, 1H), 7.60~7.52 (m, 4H), 7.50~7.42 (m, 1H), 7.24 (s, 1H), 6.97 (dd, $J=8.6$, 1.7 Hz, 1H), 6.94 (s, 1H), 6.49 (d, $J=8.6$ Hz, 1H), 4.61 (t, $J=6.9$ Hz, 2H), 4.32 (t, $J=7.4$ Hz, 2H), 4.05 (t, $J=7.1$ Hz, 2H), 2.34~2.29 (m, 2H), 1.66~1.61 (m, 2H), 1.22~1.09 (m, 2H), 0.81 (t, $J=7.3$ Hz, 3H); MS (ESI) m/z : 649 $[M+H]^+$. Anal. calcd for $C_{33}H_{29}BrN_8O_2$: C 61.02, H 4.50, N 17.25; found C 61.23, H 4.38, N 17.43.

4.2.6 Synthesis of **9**

Under a nitrogen atmosphere, **8a** (50 mg, 0.097 mmol)

was reacted with NH_4OAc (8.0 g, 104 mmol) at 140 °C for 6 h. After cooling, the resulted mixture was poured into water (50 mL), alkalinized by NaHCO_3 solution and extracted with EtOAc (50 mL $\times$ 3). The combined organic layers were washed with brine, dried over Na_2SO_4 , concentrated in vacuum and purified by flash column chromatography on silica gel using $\text{DCM}/\text{MeOH}/\text{TEA}$ ($V:V:V=120:4:1$) to give 3-(1-(3-(1*H*-imidazol-1-yl)propyl)-1*H*-indol-3-yl)-4-(1-methyl-1*H*-pyrazolo[3,4-*c*]pyridazin-3-yl)-1*H*-pyrrole-2,5-dione (**9a**) (38 mg, 86.4%) as a red solid. m.p. 108~110 °C; ^1H NMR (500 MHz, CDCl_3) δ : 11.48 (s, 1H), 9.25 (d, $J=5.6$ Hz, 1H), 8.70 (s, 1H), 8.40 (s, 1H), 8.13 (d, $J=5.6$ Hz, 1H), 7.71 (s, 1H), 7.65 (d, $J=8.2$ Hz, 1H), 7.51 (s, 1H), 7.26 (d, $J=7.6$ Hz, 1H), 6.92 (t, $J=7.6$ Hz, 1H), 6.51 (d, $J=8.1$ Hz, 1H), 4.47 (t, $J=7.4$ Hz, 2H), 4.34 (t, $J=7.2$ Hz, 2H), 4.30 (s, 3H), 2.53~2.48 (m, 2H); MS (ESI) m/z : 453 $[\text{M}+\text{H}]^+$. Anal. calcd for $\text{C}_{24}\text{H}_{20}\text{N}_8\text{O}_2$: C 63.71, H 4.46, N 24.76; found C 63.59, H 4.73, N 24.87.

3-(1-(3-(1*H*-imidazol-1-yl)propyl)-6-bromo-1*H*-indol-3-yl)-4-(1-methyl-1*H*-pyrazolo[3,4-*c*]pyridazin-3-yl)-1*H*-pyrrole-2,5-dione (**9b**): Reaction of **8b** with NH_4OAc afforded **9b** in 77.8% yield as a red solid. m.p. 115~117 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ : 11.40 (s, 1H), 9.15 (d, $J=5.6$ Hz, 1H), 8.26 (s, 1H), 8.03 (d, $J=5.6$ Hz, 1H), 7.83 (s, 1H), 7.82 (d, $J=1.8$ Hz, 1H), 7.31 (s, 1H), 7.03~6.99 (m, 2H), 6.49 (d, $J=8.6$ Hz, 1H), 4.30 (t, $J=7.4$ Hz, 2H), 4.21 (s, 3H), 4.08 (t, $J=7.2$ Hz, 2H), 2.33~2.98 (m, 2H); MS (ESI) m/z : 531 $[\text{M}+\text{H}]^+$. Anal. calcd for $\text{C}_{24}\text{H}_{19}\text{BrN}_8\text{O}_2$: C 54.25, H 3.60, N 21.09; found C 54.27, H 3.78, N 21.33.

3-(1-(3-(1*H*-imidazol-1-yl)propyl)-6-fluoro-1*H*-indol-3-yl)-4-(1-methyl-1*H*-pyrazolo[3,4-*c*]pyridazin-3-yl)-1*H*-pyrrole-2,5-dione (**9c**): Reaction of **8c** with NH_4OAc afforded **9c** in 81.5% yield as a red solid. m.p. 174~176 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ : 11.39 (s, 1H), 9.16 (d, $J=5.6$ Hz, 1H), 8.73 (s, 1H), 8.27 (s, 1H), 8.05 (d, $J=5.6$ Hz, 1H), 7.65 (s, 1H), 7.53~7.44 (m, 2H), 6.79~6.65 (m, 1H), 6.49~6.44 (m, 1H), 4.34 (t, $J=7.3$ Hz, 2H), 4.24 (t, $J=7.2$ Hz, 2H), 4.20 (s, 3H), 2.40~2.35 (m, 2H); MS (ESI) m/z : 471 $[\text{M}+\text{H}]^+$. Anal. calcd for $\text{C}_{24}\text{H}_{19}\text{FN}_8\text{O}_2$: C 61.27, H 4.07, N 23.82; found C 61.36, H 4.22, N 23.65.

3-(1-(3-(1*H*-imidazol-1-yl)propyl)-5-fluoro-1*H*-indol-3-yl)-4-(1-methyl-1*H*-pyrazolo[3,4-*c*]pyridazin-3-yl)-1*H*-pyrrole-2,5-dione (**9d**): Reaction of **8d** with NH_4OAc afforded **9d** in 79.8% yield as a red solid. m.p. 151~153 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ : 11.30 (s, 1H), 9.17 (d, $J=5.7$ Hz, 1H), 8.33 (s, 1H), 8.06 (d, $J=5.6$ Hz, 1H), 7.67 (s, 1H), 7.56~7.52 (m, 1H), 7.24 (s, 1H), 7.03 (td, $J=9.1, 2.6$ Hz, 1H), 6.94 (s, 1H), 6.29 (dd, $J=10.5, 2.6$ Hz, 1H), 4.28 (t, $J=7.3$ Hz, 2H), 4.22 (s, 3H), 4.04 (t, $J=7.2$ Hz, 2H), 2.31~2.27 (m, 2H); MS (ESI) m/z : 471 $[\text{M}+\text{H}]^+$. Anal. calcd for $\text{C}_{24}\text{H}_{19}\text{FN}_8\text{O}_2$: C 61.27, H 4.07, N 23.82; found C 61.20, H 4.10, N 23.99.

3-(1-(3-(1*H*-imidazol-1-yl)propyl)-6-methyl-1*H*-indol-3-yl)-4-(1-methyl-1*H*-pyrazolo[3,4-*c*]pyridazin-3-yl)-1*H*-pyrrole-2,5-dione (**9e**): Reaction of **8e** with NH_4OAc

afforded **8e** in 83.8% yield as a red solid. m.p. 115~117 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ : 11.31 (s, 1H), 9.12 (d, $J=5.6$ Hz, 1H), 8.21 (s, 1H), 7.99 (d, $J=5.7$ Hz, 1H), 7.95 (s, 1H), 7.36 (s, 1H), 7.27 (s, 1H), 7.08 (s, 1H), 6.63 (dd, $J=8.4, 1.5$ Hz, 1H), 6.25 (d, $J=8.2$ Hz, 1H), 4.26 (t, $J=7.3$ Hz, 2H), 4.20 (s, 3H), 4.09 (t, $J=7.1$ Hz, 2H), 2.36~2.26 (m, 5H); MS (ESI) m/z : 467 $[\text{M}+\text{H}]^+$. Anal. calcd for $\text{C}_{25}\text{H}_{22}\text{N}_8\text{O}_2$: C 64.37, H 4.75, N 24.02; found C 64.21, H 4.66, N 23.81.

3-(1-(3-(1*H*-imidazol-1-yl)propyl)-6-bromo-1*H*-indol-3-yl)-4-(1-ethyl-1*H*-pyrazolo[3,4-*c*]pyridazin-3-yl)-1*H*-pyrrole-2,5-dione (**9f**): Reaction of **8f** with NH_4OAc afforded **9f** in 82.9% yield as a red solid. m.p. 127~129 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ : 11.37 (s, 1H), 9.19 (d, $J=5.7$ Hz, 1H), 8.23 (s, 1H), 8.13 (d, $J=5.7$ Hz, 1H), 7.81 (d, $J=1.8$ Hz, 1H), 7.68 (s, 1H), 7.25 (s, 1H), 6.97 (dd, $J=8.6, 1.8$ Hz, 1H), 6.93 (s, 1H), 6.43 (d, $J=8.6$ Hz, 1H), 4.63 (q, $J=7.2$ Hz, 2H), 4.28 (t, $J=7.4$ Hz, 2H), 4.05 (t, $J=7.2$ Hz, 2H), 2.30~2.26 (m, $J=7.2$ Hz, 2H), 1.28 (t, $J=7.2$ Hz, 3H); MS (ESI) m/z : 545 $[\text{M}+\text{H}]^+$. Anal. calcd for $\text{C}_{25}\text{H}_{21}\text{BrN}_8\text{O}_2$: C 55.06, H 3.88, N 20.55; found C 54.96, H 3.64, N 20.66.

3-(1-(3-(1*H*-imidazol-1-yl)propyl)-6-bromo-1*H*-indol-3-yl)-4-(1-butyl-1*H*-pyrazolo[3,4-*c*]pyridazin-3-yl)-1*H*-pyrrole-2,5-dione (**9g**): Reaction of **8g** with NH_4OAc afforded **9g** in 87.3% yield as a red solid. m.p. 125~127 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ : 11.37 (s, 1H), 9.19 (d, $J=5.6$ Hz, 1H), 8.18 (s, 1H), 8.15 (d, $J=5.6$ Hz, 1H), 7.82 (d, $J=1.8$ Hz, 1H), 7.77 (s, 1H), 7.28 (s, 1H), 6.98 (s, 1H), 6.91 (dd, $J=8.6, 1.7$ Hz, 1H), 6.36 (d, $J=8.6$ Hz, 1H), 4.57 (t, $J=6.9$ Hz, 2H), 4.29 (t, $J=7.4$ Hz, 2H), 4.06 (t, $J=7.2$ Hz, 2H), 2.31~2.27 (m, 2H), 1.62~1.57 (m, 2H), 1.16~1.08 (m, 2H), 0.79 (t, $J=7.4$ Hz, 3H); MS (ESI) m/z : 545 $[\text{M}+\text{H}]^+$. Anal. calcd for $\text{C}_{27}\text{H}_{25}\text{BrN}_8\text{O}_2$: C 56.55, H 4.39, N 19.54; found C 56.81, H 4.51, N 19.73.

3-(6-bromo-1*H*-indol-3-yl)-4-(1-methyl-1*H*-pyrazolo[3,4-*c*]pyridazin-3-yl)-1*H*-pyrrole-2,5-dione (**9h**): Reaction of **7b** with NH_4OAc afforded **9h** in 81.2% yield as a red solid. m.p. >250 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ : 12.10 (s, 1H), 11.34 (s, 1H), 9.15 (d, $J=5.6$ Hz, 1H), 8.22 (d, $J=2.9$ Hz, 1H), 8.01 (d, $J=5.6$ Hz, 1H), 7.64 (d, $J=1.9$ Hz, 1H), 6.93 (dd, $J=8.6, 1.9$ Hz, 1H), 6.37 (d, $J=8.7$ Hz, 1H), 4.21 (s, 3H); MS (ESI) m/z : 423 $[\text{M}+\text{H}]^+$. Anal. calcd for $\text{C}_{18}\text{H}_{11}\text{BrN}_6\text{O}_2$: C 51.08, H 2.62, N 19.86; found C 51.22, H 2.54, N 19.66.

3-(1-(3-(1*H*-imidazol-1-yl)propyl)-6-bromo-1*H*-indol-3-yl)-4-(1-methyl-1*H*-pyrazolo[3,4-*c*]pyridazin-3-yl)-1-methyl-1*H*-pyrrole-2,5-dione (**9i**): Compound **8b** (30.0 mg, 0.049 mmol) was reacted with a solution of CH_3NH_2 in methanol (30%, 30 mL) at room temperature for 2 h. The solvent was concentrated in vacuum and the residue was purified by flash column chromatography on silica gel using $\text{DCM}/\text{MeOH}/\text{TEA}$ ($V:V:V=120:4:1$) to afford **9i** (22 mg, 81.5%) as a red solid. m.p. 144~146 °C; ^1H NMR (500 MHz, $\text{DMSO}-d_6$) δ : 9.16 (d, $J=5.6$ Hz, 1H), 8.30 (s, 1H), 8.03 (d, $J=5.6$ Hz, 1H), 7.83 (d, $J=1.8$ Hz, 1H), 7.69 (s, 1H), 7.28~7.23 (m, 1H), 7.03 (dd, $J=8.6, 1.7$ Hz, 1H),

6.94 (s, 1H), 6.56 (d, $J=8.7$ Hz, 1H), 4.30 (t, $J=7.3$ Hz, 2H), 4.22 (s, 3H), 4.05 (t, $J=7.2$ Hz, 2H), 3.10 (s, 3H), 2.32~2.25 (m, 2H); MS (ESI) m/z : 545 $[M+H]^+$. Anal. calcd for $C_{25}H_{21}BrN_8O_2$: C 55.06, H 3.88, N 20.55; found C 55.21, H 3.63, N 20.71.

4.3 Biochemical assays

4.3.1 IDH1 mutations inhibition assay

All recombinant human IDH1 mutations protein (IDH1/R132H, IDH1/R132C and IDH1/R132L), fused at the N-terminus to GST, were expressed in *E. coli* and purified using GST affinity column chromatography (QIAGEN). Enzyme inhibition assay for IDH1 mutations were performed in a 384-well microplate in 25 mmol/L Tris-HCl buffer (pH 7.0) containing the enzyme (20~40 nmol/L), 8 mmol/L $MnCl_2$, 12 μ mol/L NADPH, and compounds. Upon incubation for 15 min, 1 mmol/L α -KG was added to initiate the reaction. And the decrease of NADPH was detected by Envision, at 355 nm excitation and 460 nm Emission (PerkinElmer). The IC_{50} data was calculated using the software GraphPad Prism, and chosen the equation "sigmoidal dose-response (variable slope)" for curve fitting.

4.3.2 IDH1/WT inhibition assay

Recombinant human IDH1/WT fused at the N-terminus to GST and expressed. The inhibition assay of IDH1/WT was carried out in 25 mmol/L Tris-HCl buffer (pH 7.0) containing the enzyme (30 nmol/L), 1 mmol/L $MgCl_2$, 75 μ mol/L NADP, an inhibitor, and 100 μ mol/L sodium (*D*)-isocitrate. And the NADPH of probe was detected by monitoring the increase of fluorescence with Envision (PerkinElmer), at 355 nm excitation and 460 nm Emission. The IC_{50} data was calculated using the software GraphPad Prism, and chosen the equation "sigmoidal dose-response (variable slope)" for curve fitting.

4.3.3 U87MG IDH1-R132H-puro Cell-based Assays and LC-MS/MS measurement of 2-HG

R132H mutations were introduced into human IDH1 by standard molecular biology techniques. Human glioma U87MG cell lines were transfected using standard techniques. U87MG IDH1-R132H-puro cells were maintained in DMEM containing, 10% FBS, 100 U/mL penicillin and 100 μ g/mL streptomycin. Cells were seeded at a density of 20000 cells/well into 96-well microtiter plates and incubated overnight at 37 °C and 5% CO_2 . The next day compounds were prepared in 100% dimethyl sulfoxide (DMSO) and then diluted in medium for a final concentration of 0.2% DMSO. Medium was removed from the cell plates, and 200 μ L of the compound dilutions were added to each well. After 48 h of incubation with compound at 37 °C, medium was removed from each well and pelleted cells were washed twice with ice-cold PBS before sequential quenching with -80 °C 80% methanol/20% water. Intracellular 2-HG and L-glutamic acid were measured by LC-MS/MS.

Supporting Information 1H NMR of compounds 2~9i. The Supporting Information is available free of charge via

the Internet at <http://sioc-journal.cn>.

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