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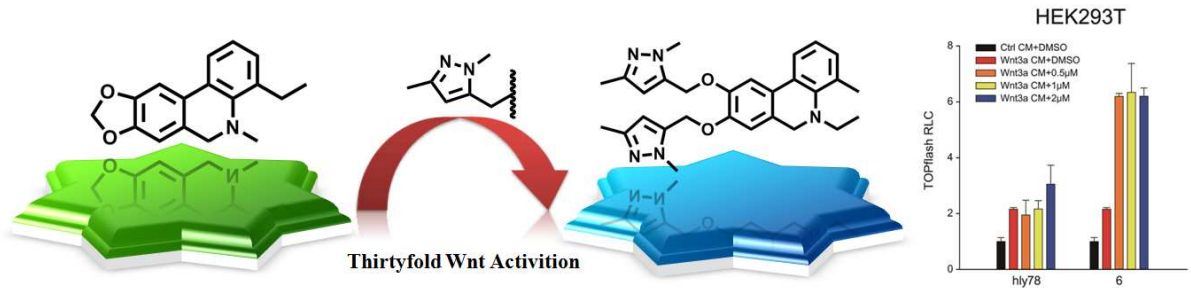

Efficient Synthesis of New Phenanthridine Wnt/*β*-Catenin Signaling Pathway Agonists

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Abstract

Previously, HLY78, a lycorine derivative, was identified as the first Wnt/***β***-catenin signaling agonist through binding to the DAX domain of Axin, a scaffold of Wnt/***β***-catenin complex. In this study, to obtain more potent Wnt/***β***-catenin agonist, the structure optimization of HLY78 was carried out by design and synthesis of six phenanthridine derivatives, which afforded five active ones. In particular, 8,9-bis((1,3-dimethyl-1H-pyrazol)methoxy)-5-ethyl-4-methyl-5,6-dihydrophenanthridine showed the most potent activity (0.15 /μM) that was increased nearly 30 times as that of the lead HLY78. These compounds may be valuable in future pharmacological or biological studies.

Key words.

Wnt/***β***-catenin signaling agonist; Phenanthridine derivatives; Structural optimization

1. Introduction

Wnt/***β***-catenin is a highly conserved signaling pathway[1,2] that is important for development, especially for embryonic development. Wnt regulates the processes of axis patterning, cell fate specification, cell proliferation and cell migration. Aberrant Wnt signaling involves in many diseases, such as cancers (in which the pathway is inappropriately activated), Alzheimer's disease and osteoporosis (in which the pathway is attenuated).[3-5] In recent years, Wnt antagonists have attracted attention due to potential applications in the clinical treatment of cancers.[6-10] Appropriate activation of Wnt signaling pathways could also be useful in other clinical applications, such as hematopoietic stem cell (HSC) expansion and the treatment of osteoporosis.[11] However, Wnt-based new drug research has developed slowly because of a lack of

effective, safe and Wnt-specific small molecule agonists.

A new small-molecule activator of the Wnt/***β***-catenin signaling pathway, 4-ethyl-5-methyl-5,6-dihydro-[1,3]-dioxolo-[4,5-j]-phenanthridine (HLY78, Figure 1), has previously been identified. Mechanistic studies have shown that HLY78 binds to the DAX domain of the Axin protein and potentiates the Axin/LRP6 association, which subsequently promotes LRP6 phosphorylation and Wnt signaling transduction.[12]

HLY78 has been previously demonstrated as a Wnt-specific small molecule, and the activation mechanism of HLY78 that affects the stability of the Wnt transcribing complex makesHLY78 safe for potential pharmacological use. However, HLY78 has a disadvantage in that its activity is too low to be used in future pharmacological studies. To identify an effective Wnt agonist suitable for pharmacological use, the structure of HLY78 must be further optimized using a rational drug design approach. We performed a preliminary structural optimization of HLY78 and analyses of the structure-activity relationships (SARs) of phenanthridine derivatives as agonists of the Wnt/***β***-catenin signaling pathway. In these studies, several satisfactory Wnt agonists were achieved, and a number of potentially active groups, such as the triazole group, were discovered.[13] Even though the Wnt activation of certain phenanthridine derivatives was clearly increased, a disadvantage was that the activity of certain compounds was still come lower than an ideal level, and all of these compounds were created through a semi-synthetic procedure beginning with lycorine, meaning that all of the synthesized compounds were substituted at the same positions. We developed a synthesis procedure for a complete phenanthridine skeleton for the purpose of evaluating compounds with different substituents.

In this report, an efficient discovery process for several good Wnt/***β***-catenin signaling agonists was presented and a systematic and in-depth structural optimization of several phenanthridine analogues based on previous studies of structure-activity relationships (SARs) was profermed. The *in vitro* Wnt-activating effects of these derivatives and the target protein were also evaluated.

2. Results and Discussion

2.1 Simulation of Potential Active Groups of Potent Wnt Agonists by "Model Molecules".

In a previous study of the Wnt agonists, a phenanthridine derivative (HLYC60, Figure 1) that exhibited strong Wnt activation was designed, and the study further suggested that the triazole group is an important functional group for Wnt activation, i.e., the combination of a 4-ethyldihydrophenanthridine skeleton and triazole substituents improves Wnt activation. [13] However, according to a docking analysis of binding sites of 4-ethyldihydro-phenanthridine in Axin, an ethyl group at the C - 4 position seems to adversely affect the entrance of compounds into the narrow cavity of the binding site. In addition, the introduction of a triazole moiety into the phenanthridine skeleton involves a relatively complex click reaction between an azide and a propargyl phenanthridine, and the results of an *in vitro* assay indicate that it causes the concomitant introduction of an ethylamine group, which seems to decrease the acrivition of compounds.

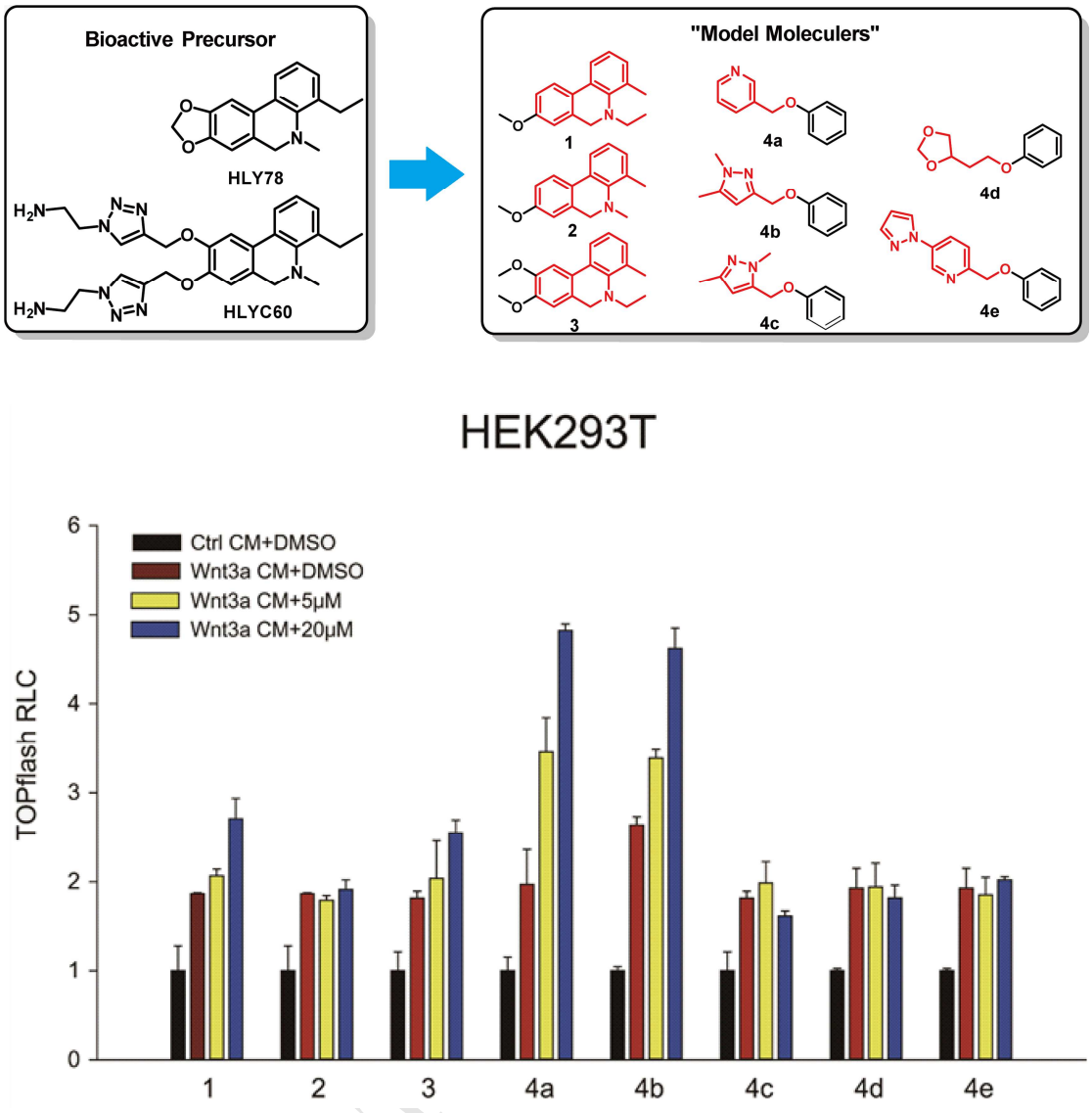


Fig. 1. Design synthesis and bioassay of "model molecules." C-4 methyl, N-ethyl, pyrazole and pyridine moiety (red labeled) could enhance the activation of Wnt signaling.

As previously mentioned, the phenanthridine skeleton and triazole require further optimization. Thus, we planned to modify the C-4 and N-5 substituents of the phenanthridine skeleton by replacing the original triazole groups with pyrazole or pyridine substituents, which can be easily introduced by a classical alkylation reaction. To confirm the effect of such optimization, a series of “model molecules” was designed and synthesized to simulate different C-4 and N-5 substituted phenanthridine skeletons, as well as different pyrazole and pyridine substructures (Figure 1). Of these

molecules, compounds **1**, **2**, and **3** were used to study the effects of the various C-4 and N-5 substituents, and compounds **4a-e** were used to investigate the effects of the pyrazole or pyridine groups. The capacity of these compounds to activate Wnt signaling was later investigated. Compounds **1**, **3**, **4a**, **4b**, and **4c** could active Wnt within the tested concentrations from 5-20μM, implying that a C-4 methyl, N-ethyl, pyrazole, or pyridine moiety could enhance the activation of Wnt signaling.

2.2 Design of Potential Phenanthridine Wnt Agonists.

The previous studies prompted the creation of compounds containing C-4 methyl or N-ethyl groups and pyrazole or pyridine substituents at the free C-8 and C-9 hydroxy groups to optimize the activity of these compounds. We designed compounds **5**, **6**, **7**, **8** and **9** to further investigate compounds with these features (Figure 2). Previous studies have demonstrated that the bioactive precursor of these compounds, HLY78, actives Wnt by targeting at dix domain of Axin protein. [12] Therefore, before these phenanthridine derivatives were actually synthesized, docking analyses of **5**, **6**, **7**, **8** and **9** were conducted in order to predict their activity on the Wnt signaling pathway (Figure 3). The well-characterized X-ray crystal structure of Axin (PDB 1WSP) [14] and of compound **5**, **6**, **7**, **8** and **9** were investigated to predict the binding mode, and the molecular docking of the compounds into the Axin DAX binding domain was analyzed with AutoDock [15] to understand the interactions between these compounds and Axin. All ligand docking conformations vis a vis the DAX domain were analyzed, and the model with the lowest estimated free energy for binding was selected. The modeled Axin-ligand complex structurerevealed that a group of residues in the cavity are in contact with the phenanthridine derivatives via electrostatic or hydrophobic interactions. The docking results predicted that a pyrazole and pyridine substituent at the C-8 and C-9 position may caused a tighter integration between the Axin molecule and the phenanthridine compound.

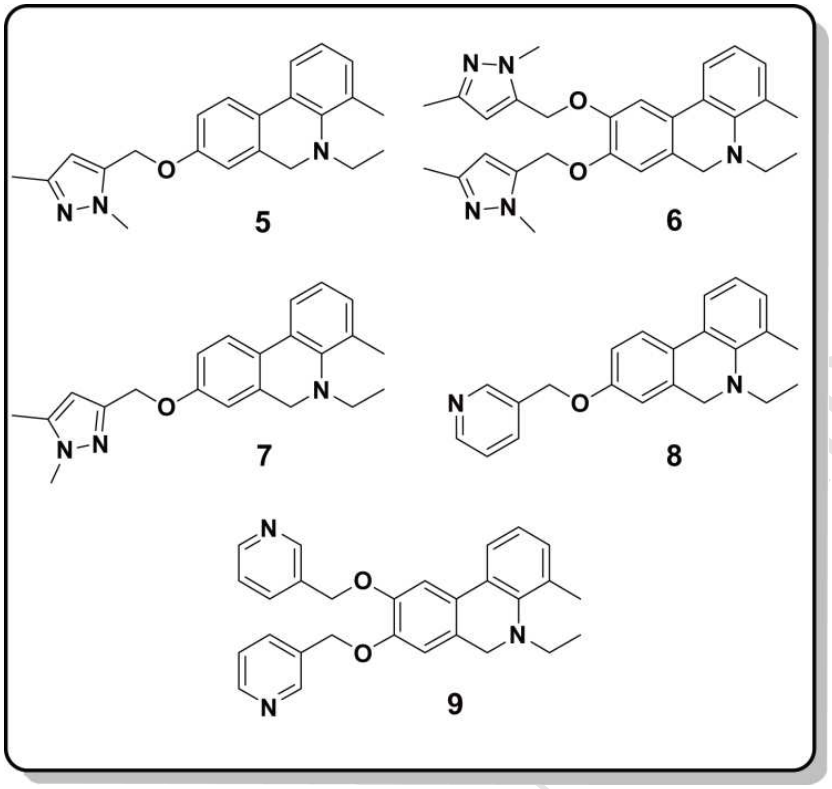


Fig. 2. Designed potential phenanthridine. According to the studies on "model molecules", compounds that containing C-4 methyl or N-ethyl groups and pyrazole or pyridine substituents at the C-8 and C-9 hydroxy groups were designed.

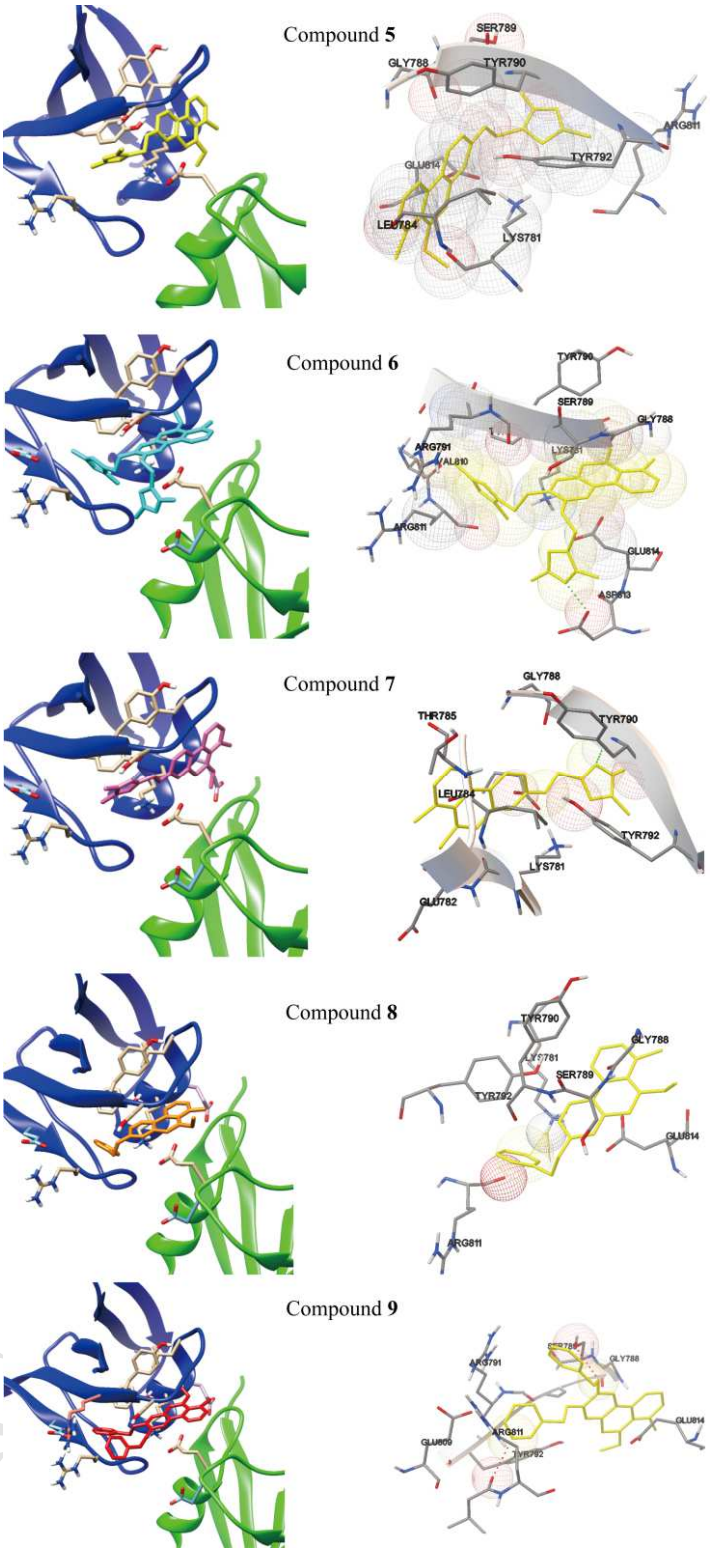
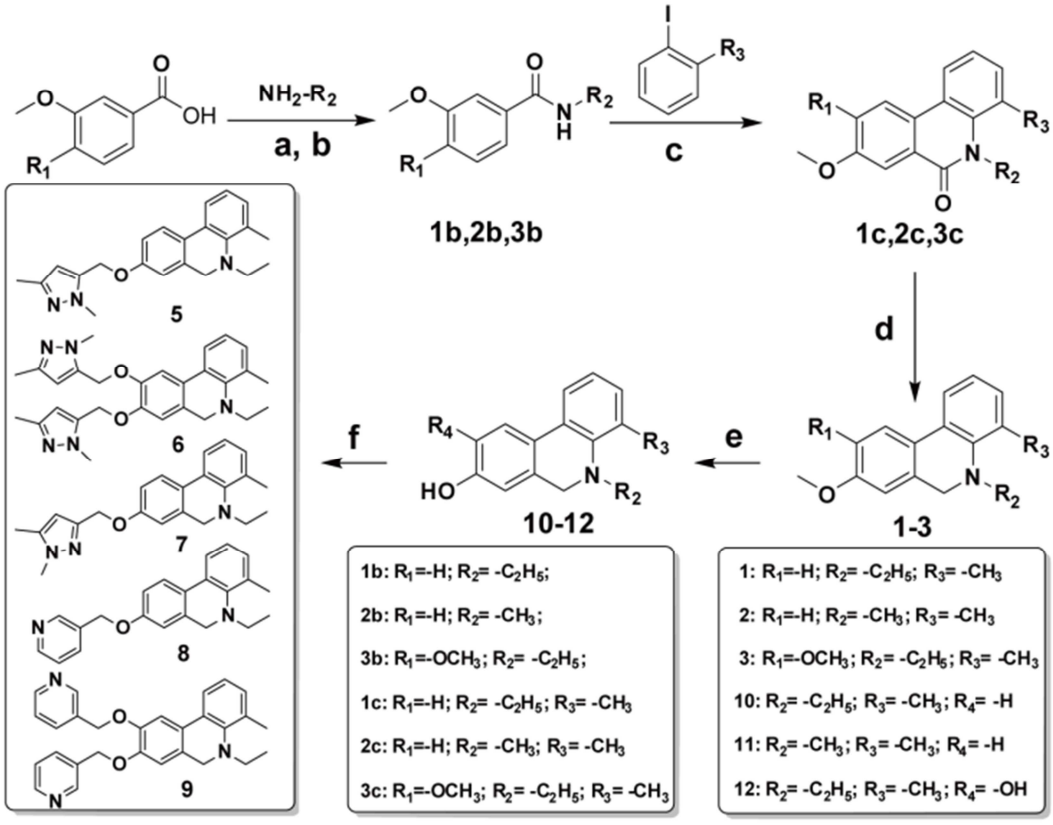


Fig. 3. Wnt agonists and schematic representations of their docking with the crystal structure of DAX (PDB 1WSP) are shown. The estimated binding free energies are: -7.17 kcal/mol (**5**), -8.23 kcal/mol (**6**), -9.42 kcal/mol (**7**), -6.75 kcal/mol (**8**) and -7.03 kcal/mol (**9**).

2.3 Synthesis of Potential Phenanthridine Wnt Agonists.

On the basis of the previous results, **5**, **6**, **7**, **8** and **9** were actually synthesized via a total synthesis procedure. First, three initial compounds (**1**, **2** and **3**) were respectively generated from 2-bromo-5-methoxybenzoic (**1a**) acid or 2-bromo-4,5-dimethoxybenzoic acid (**3a**) in four steps. Compound **1a** or **3a** was amidated with ethylamine to yield **1b**, **2b** or **3b**, which was later condensed with 2-methyliodobenzene or 2-ethyliodobenzene using palladium catalysis to yield **1c**, **2c** or **3c**, followed by the reduction of the C-6 carbonyl to yield**1**, **2** or **3**. Finally, compounds**10**, **11** or **12**were respectively obtained by the deprotection of **1**, **2** or **3** in the presence of boron tribromide. These initial compounds were subsequently alkylated using different alkylating agents to yield the phenanthridine derivatives **5**, **6**, **7**, **8** and **9** (Scheme1).



Scheme 1. Synthesis of potential phenanthridine Wnt agonists. [a]

[a] **Reagents and conditions:** (a) $SOCl_2$, DMF, THF, 50 °C, 2 h; (b) 5 °C, 1 h; (c) K_2CO_3, norbornene, $Pd(OAc)_2$, TFP, MeCN, 85 °C, 6 h; (d) BH_3-THF, THF, -78 °C, 2

h; (e). BBr_3, CH_2Cl_2, -78 °C, 4 h; f. NaH, alkylating agent, CH_2Cl_2 or THF, 60-100 °C, 6-18h

2.4 Activation of the Wnt/β-catenin Signaling Pathway by Phenanthridine Derivatives.

The effects of all of the derivatives on a reporter gene of Wnt/β-catenin signaling pathway activity were evaluated in HEK293T cells. This protocol can be adapted to screen small molecules that either increase or decrease the Wnt pathway response. Canonical Wnt signaling activity transcriptionally induces Wnt target genes that contain consensus TCF/LEF binding elements. Wnt pathway responsive cells that transiently or stably express luciferase proteins under the TCF/LEF promoter can be used to detect stimulus-dependent Wnt-pathway activity. As expected, compounds **5**, **6**, **7** and **8** clearly activated Wnt signaling in the reporter gene assay, and **6** exhibited the highest Wnt agonist activity of the synthetic phenanthridine derivatives. Compound **6** doubled the activation of the Wnt signaling pathway at the low concentration of 0.15 μM (Figure 4a) and is by far the most active phenanthridine Wnt agonist which targets Axin. To further confirm the activation function, the effects of compounds **6** and **7** on the expression of two endogenous Wnt target genes (i.e., Axin2 and DKK1) were tested. These compounds could up-regulate the expression of these endogenous Wnt target genes at a concentration of 0.5 μM and 5 μM, respectively. (Figure 4b).

2.5 Phenanthridine Derivatives Target the Axin DAX Domain.

With the purpose to investigate if the phenanthridine derivatives also activate Wnt signaling pathway by targeting Axin as HLY78 does, an *in vitro* analysis using a purified recombinant DAX protein was conducted and it indicated that the DAX domain of Axin was a direct target of designed phenanthridine derivatives **5**, **6**, **7** and **9** The binding between DAX and HLY179 (HLY78 bears biotin chain [12]) could be specifically competed out these compounds (Figure 4c).

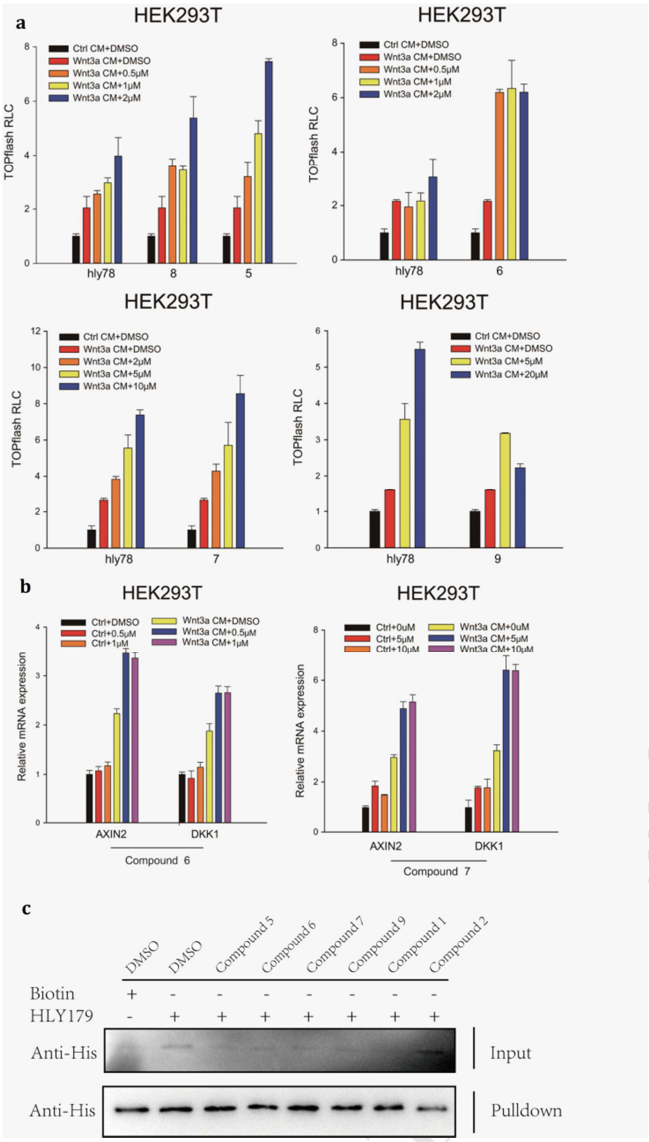


Fig. 4. Activation of the Wnt/***β***-catenin Signaling Pathway by Phenanthridine Derivatives a. Wnt signaling activation of phenanthridine derivatives in a reporter gene assay. Compounds **5**, **6**, **7** and **8** could activate Wnt signaling in a reporter gene assay, and compound **6** exhibits the strongest activity. b. Wnt activation of phenanthridine derivatives on the expression of the endogenous Wnt target genes Axin2 and DKK1. Compounds **6** and **7** clearly activated the expression of the endogenous Wnt target genes Axin2 and DKK1. c. Phenanthridine derivatives directly bind the Axin DAX domain. Immunoblotting analysis on streptavidin-coated Sepharose after incubation of the purified recombinant DAX with HLY179 (20 μM) or biotin in the presence of Phenanthridine Derivatives **8** (80 μM).

2.6 SAR Analysis of Phenanthridine Derivatives.

As Table 1 shows, the analogues, **5**,**6**, and **7** exhibit stronger Wnt activating activity than HLY78 does, meanwhile, the docking results also shows that pyrazole groups could adhered well to residue of DAX domain, these suggests that the introduction of pyrazole groups at C-8 and C-9 enhances the activity of the phenanthridine derivatives. Furthermore, in comparison with HLYC60, pyrazole groups can be easily introduced through a simple alkylation reaction instead of the relatively complex click reaction which is used to introduce a triazole group. In addition, that compound **8** exhibits stronger activity than compound **9,** which activated Wnt at the same level as HLY78, implied that the effects of pyridine disubstituents at C-8 and C-9 in terms of the improved activation of Wnt signaling were limited. This prediction has also been supported by docking results, pyridine disubstituents would result in higher binding energy which makes the connection between compounds and protein more unstable.

Table 1. Wnt Active activity of compounds **5-9**

Compounds	[α] Concentration that Doubled the Activation of Wnt
3 (HLY78)	5.00 ±0.6μM
5	0.50 ± 0.1 μM
6	0.15 ± 0.02 μM
7	3.00 ± 0.9 μM
8	1.50 ± 0.2 μM
9	5.00 ± 0.7 μM

[α] The minimum concentration that compounds could at least double the activation of Wnt signaling pathway.

3. Conclusions

In conclusion, previous studies efficiently achieved good Wnt agonists through the synthesis of relatively small amounts of derivatives In addition, this result indicates that the combination of a 4-methyl,N-ethyl-dihydrophenanthridine skeleton and pyrazole and pyridine substituents enhances Wnt activation, and compounds **6** and **5** are the most suitable for further pharmacological or biological studies.

4. Experimental Procedures

4.1 Chemical

General Experimental Procedures

Melting points were measured using an X-4 apparatus (Yingyu Yuhua Instrument Factory, Gongyi, Henan Province, P. R. China). ESI and HRMS data were recorded using Finnigan MAT 90 instrument and VG Auto Spec-3000 spectrometer, respectively. NMR experiments were conducted on Bruker AM-400, DRX-500, or Avance III 600 spectrometer using residual $CDCl_3$ and DMSO-d_6 or TMS as internal standards. Column chromatography was performed on silica gel (60-80 mesh, 200-300 mesh, 300-400 mesh, Qingdao Haiyang Chemical Co. Ltd., Qingdao, P. R.China)**.** Pre-coated silica gel 60 GF254 (Merck, Darmstadt, Germany) was used for TLC analyses. Semipreparative HPLC analyses were performed on a Hypersil Gold RP-C_{18} column (i.d. 10 × 250 mm, 5 μm, 5 mL/min) developed with CH_3CN-H_2O at room temperature (r.t.). All regular solvents and reagents were reagent grade and were purchased from Sigma-Aldrich Chemical Co. (St. Louis, United States), Acros Organics (Geel, Belgium), and J&K Scientific (Beijing, P. R.China). The purities of all compounds used in biological assays exceeded 95%, as determined by HPLC. HPLC was performed on an X-Bridge RP-C_{18} column (4.6 × 250 mm, 5 μm, 5 mL/min) with CH_3OH-H_2O at r.t. All reported yields are for dry compounds that required no further purification for use in other reactions. Each bioassay experiment was repeated at least three times.

Syntheses of Phenanthridine Compounds

2-Bromo-N-ethyl-4-methoxybenzamide (**1b**)**.** Compound **1a** (260 mg, 1mmol) was dissolved in THF (10 mL), to which DMF (0.1 mL) and $SOCl_2$ (0.5 mL, 4mmol) were added. The reaction solution was stirred for 2 h at 50 °C and then concentrated to remove THF. The residue was then added to a 30% solution of ethylamine in water (20 mL) at 5°C and filtered. The cake was purified by column chromatography to give **4** as a pale yellow solid (205 mg, 75 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43 (d,

J = 8.8 Hz, 1H), 7.08 (d, *J* = 3.1 Hz, 1H), 6.81 (dd, *J* = 8.8, 3.1 Hz, 1H), 3.80 (s, 3H), 3.49 (q, *J* = 7.0 Hz, 2H), 1.26 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.23 (C), 158.94 (C), 138.53 (C), 134.13 (CH), 117.81 (CH), 114.63 (CH), 109.35 (C), 55.66 (CH_3), 35.06 (CH_2), 14.69 (CH_3); HREIMS *m/z* 257.0050 $[M]^+$ (calcd for $C_{10}H_{12}BrNO_2$, 257.0051).

4-Methyl-N-ethyl-8-methoxyphenanthridin-6(5*H*)-one (1c). A flask was charged under nitrogen with $Pd(OAc)_2$ (3.0 mg, 0.013 mmol), tri-2-furylphosphine (6.2 mg, 0.027 mmol), K_2CO_3 (72.3 mg, 0.52 mmol), the amide **1b** (0.26 mmol), a solution of norbornene (26.9 mg, 0.286 mmol) in anhydrous solvent (5.8 mL), and 1-iodo-2-methylbenzene (0.26 mmol). The reaction mixture was heated with stirring at 85 °C for 6 h and then cooled to r. t. After the addition of saturated NH_4Cl (30 mL) and extraction with EtOAc (3×15 mL), the combined organic extracts were washed with brine (30 mL) and dried over Na_2SO_4. Removal of the solvent under reduced pressure gave the crude product, which was purified by flash chromatography on silica gel to furnish **1c** as white wax (55 mg, 80 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.15 (d, *J* = 9.0 Hz, 1H), 8.06 (d, *J* = 8.2 Hz, 1H), 7.92 (d, *J* = 2.8 Hz, 1H), 7.32 (dd, *J* = 8.9, 2.8 Hz, 1H), 7.28 (d, *J* = 7.2 Hz, 1H), 7.20 (t, *J* = 7.7 Hz, 1H), 4.51 (q, *J* = 6.9 Hz, 2H), 3.96 (s, 3H), 2.70 (s, 3H), 1.37 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 163.90 (C), 159.37 (C), 137.15 (C), 133.12 (CH), 127.56 (C), 126.84 (C), 125.82 (C), 123.69 (CH), 122.74 (CH), 122.37 (CH), 121.81 (C), 120.90 (CH), 108.76 (CH), 55.64 (CH_3), 42.60 (CH_2), 23.87 (CH_3), 14.83 (CH_3); HREIMS *m/z* 267.1263 $[M]^+$ (calcd for $C_{17}H_{17}NO_2$, 267.1259).

4-Methyl-N-ethyl-8-methoxy-5,6-dihydrophenanthridine (1). A solution of **1c** (30 mg, 0.1 mmol) in THF (5 mL) was added to BH_3-THF (1M, 1 mL) at -78 °C. The reaction was stirred for 2 h and then quenched using H_2O (5 mL). The mixture was t;lhen extracted with Et_2O (20 mL) twice. The organic phase was washed with brine and concentrated, and the residue was purified by column chromatography to give **1** as a colorless solid (18 mg, 70 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.67 (d, *J* = 8.5 Hz, 1H), 7.58 (d, *J* = 7.5 Hz, 1H), 7.13 (d, *J* = 6.6 Hz, 1H), 7.08 (t, *J* = 7.5 Hz,

1H), 6.90 (dd, *J* = 8.5, 2.6 Hz, 1H), 6.77 (d, *J* = 2.6 Hz, 1H), 4.10 (s, 2H), 3.86 (s, 3H), 2.70 (q, *J* = 7.1 Hz, 2H), 2.37 (s, 3H), 1.10 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.30 (C), 146.27 (C), 135.05 (C), 133.38 (C), 129.69 (CH), 129.38 (C), 125.87 (C), 124.31 (CH), 123.86 (CH), 120.83 (CH), 112.92 (CH), 111.92 (CH), 55.33 (CH_3), 50.25 (CH_2), 46.11 (CH_2), 17.81 (CH_3), 13.64 (CH_3); HRESIMS *m/z* 254.1539 $[M+H]^+$ (calcd for $C_{17}H_{20}NO$, 254.1539).

2-Bromo-4-methoxy-N-methyl-Benzenemethanamine (**2b**)**.** By referencing the synthesis method of compound **1b**, compound **2b** was conducted by amidating of compound **1a** at the presence of 30% solution of methylamine (20 mL) in a yield of 72% (200 mg). m.p. 152 ℃。^{1}H NMR (400MHz, $CDCl_3$) δ 7.43 (d, *J* = 8.8 Hz, 1H) , 7.07 (d, *J* = 3.1 Hz, 1H) , 6.81 (dd, *J* = 3.1, 8.8 Hz, 1H) , 3.79 (s, 3H) , 3.00 (s, 3H) ; ^{13}C NMR (100MHz, $CDCl_3$) δ 168.0 (C), 158.8 (C), 138.3 (C), 134.1 (CH), 117.8 (CH), 114.6 (CH), 109.3 (C), 55.6 (CH_3), 26.7 (CH_3); HREIMS *m/z* 242.9889 $[M]^+$ (calcd for $C_9H_{10}BrNO_2$, 242.9895).

4-Methyl-N-methyl-8-methoxyphenanthridin-6(5*H*)-one (2c). By referencing the synthesis method of compound **1c**, compound **2b** (0.1mmol) and 1-iodo-2-methylbenzene (0.12 mol) coupled to afford compound **2c** (68 % yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.13 (d, *J* = 9.0 Hz, 1H), 8.01 (d, *J* = 7.8 Hz, 1H), 7.91 (d, *J* = 2.8 Hz, 1H), 7.31 (dd, *J* = 8.9, 2.8 Hz, 1H), 7.28 – 7.23 (m, 1H), 7.18 (d, *J* = 7.5 Hz, 1H), 3.95 (s, 3H), 3.81 (s, 3H), 2.66 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.99 (C), 159.42 (C), 138.5 (C), 132.77 (CH), 127.54 (C), 126.67 (C), 126.12 (C), 123.74 (CH), 122.31 (CH), 122.9 (CH), 121.38 (C), 120.50 (CH), 108.80 (CH), 55.71 (CH_3), 38.51 (CH_3), 23.63 (CH_3); HREIMS *m/z* 253.1102 $[M]^+$ (calcd for $C_{16}H_{15}NO_2$, 253.1103).

4-Methyl-N-methyl-8-methoxy-5,6-dihydrophenanthridine (2). By referencing the synthesis method of compound **1**, compound **2** was conducted by reduction of compound **2c** (0.1 mmol) at the presence of BH_3-THF (1M, 0.25 mmol) in a yield of 72%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.70 (d, *J* = 8.6 Hz, 1H), 7.61 (dd, *J* = 7.5, 1.5 Hz, 1H), 7.18 – 7.08 (m, 2H), 6.93 (dd, *J* = 8.5, 2.7 Hz, 1H), 6.81 (d, *J* = 2.6 Hz, 1H), 4.09

(s, 2H), 3.88 (s, 3H), 2.52 (s, 3H), 2.43 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.33 (C), 145.89 (C), 134.22 (C), 133.30 (C), 129.53 (CH), 125.47 (C), 125.14 (C), 124.26 (CH), 124.19 (CH), 120.93 (CH), 113.02 (CH), 112.01 (CH), 55.39 (CH_2), 55.21 (CH_3), 40.37 (CH_3), 17.50 (CH_3); HRESIMS *m/z* 240.1385 $[M+H]^+$ (calcd for $C_{16}H_{18}NO$, 240.1383).

2-Bromo-4,5-methoxy-N-ethyl-Benzenemethanamine (**3b**)**.** By referencing the synthesis method of compound **1b**, compound **3b** was conducted by amidating of compound **3a** (1 mmol) at the presence of ethylamine (50mL) in a yield of 85%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20 (s, 1H), 6.98 (s, 1H), 3.89 (s, 3H), 3.89 (s, 3H), 3.50 (q, *J* = 7.2 Hz, 2H), 1.27 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.72 (C), 150.69 (C), 148.42 (C), 129.27 (C), 115.67 (CH), 112.90 (CH), 109.70 (C), 56.29 (CH_3), 56.15 (CH_3), 35.13 (CH_2), 14.70 (CH_3); HREIMS *m/z* 287.0157 $[M]^+$ (calcd for $C_{11}H_{14}BrNO_3$,287.0157).

4-Methyl-N-ethyl-8,9-dimethoxyphenanthridin-6(5*H*)-one (3c). By referencing the synthesis method of compound **1c**, compound **3b** (0.1 mmol) and 1-iodo-2-methylbenzene (0.12mmol) coupled to afford compound **3c** (75 % yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.97 (d, *J* = 7.8 Hz, 1H), 7.87 (s, 1H), 7.52 (s, 1H), 7.26 (dd, *J* = 5.1, 2.1 Hz, 1H), 7.16 (t, *J* = 7.6 Hz, 1H), 4.47 (q, *J* = 7.0 Hz, 2H), 4.05 (s, 3H), 4.01 (s, 3H), 2.68 (s, 3H), 1.34 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.64 (C), 153.27 (C), 149.69 (C), 137.81 (C), 133.36 (CH), 128.75 (C), 125.90 (C), 122.49 (CH), 121.53 (C), 120.93 (CH), 119.64 (C), 108.75 (CH), 102.83 (CH), 56.17 (CH_3), 56.10 (CH_3), 42.45 (CH_2), 23.95 (CH_3), 14.97 (CH_3); HRESIMS *m/z* 298.1441 $[M+H]^+$ (calcd for $C_{18}H_{20}NO_3$, 298.1438).

4-Methyl-N-ethyl-8,9-dimethoxy-5,6-dihydrophenanthridine (3). By referencing the synthesis method of compound **1**, compound **3** was conducted by reduction of compound **3c** (0.1mmol) at the presence of BH_3-THF (1M, 0.3mmol) in a yield of 65%. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 (d, *J* = 6.9 Hz, 1H), 7.24 (s, 1H), 7.12 (d, *J* = 7.4 Hz, 1H), 7.07 (t, *J* = 7.5 Hz, 1H), 6.73 (s, 1H), 4.06 (s, 2H), 3.96 (s, 3H), 3.93 (s, 3H), 2.70 (q, *J* = 7.1 Hz, 2H), 2.36 (s, 3H), 1.09 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (125

MHz, $CDCl_3$) δ 148.43 (C), 146.36 (C), 140.87 (C), 133.45 (C), 129.81 (CH), 129.38 (C), 125.95 (C), 125.47 (C), 123.75 (CH), 120.70 (CH), 109.64 (CH), 106.54 (CH), 56.12 (CH_3), 56.01 (CH_3), 49.65 (CH_2), 45.96 (CH_2), 17.83 (CH_3), 13.69 (CH_3); HRESIMS *m/z* 284.1648 $[M+H]^+$ (calcd for $C_{18}H_{22}NO_2$, 284.1645).

Syntheses of Compounds 10, **11**, and **12**. Compound **1, 2** or **3** (0.1 mmol) was dissolved in 10 mL CH_2Cl_2. The reaction solution was then cooled to -78 °C and BBr_3 (100 μL, 0.2 mmol) was added. The mixture was then stirred for 4 h after which it was diluted in 10 mL saturated $NaHCO_3$. The solution was extracted twice with CH_2Cl_2 (15 mL), and the organic layer was washed with brine, concentrated, and then purified by column chromatography using chloroform-methanol (20:1) as the eluent to yield compounds **10, 11** and **12**, respectively.

5-ethyl-4-methyl-5,6-dihydrophenanthridin-8-ol (10). 72% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (d, *J* = 8.3 Hz, 1H), 7.56 (d, *J* = 7.5 Hz, 1H), 7.12 (d, *J* = 7.3 Hz, 1H), 7.07 (t, *J* = 7.5 Hz, 1H), 6.81 (dd, *J* = 8.3, 2.4 Hz, 1H), 6.71 (d, *J* = 2.2 Hz, 1H), 4.05 (s, 2H), 2.69 (q, *J* = 7.1 Hz, 2H), 2.36 (s, 3H), 1.08 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 155.29 (C), 146.18 (C), 135.33 (C), 133.38 (C), 129.71 (CH), 129.32 (C), 125.99 (C), 124.51 (CH), 123.87 (CH), 120.78 (CH), 114.42 (CH), 113.39 (CH), 50.02 (CH_2), 46.08 (CH_2), 17.79 (CH_3), 13.59 (CH_3); HRESIMS *m/z* 240.1386 $[M+H]^+$ (calcd for $C_{16}H_{18}NO$, 240.1383).

4,5-dimethyl-5,6-dihydrophenanthridin-8-ol (11). 68% yield; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 (d, *J* = 8.4 Hz, 1H), 7.57 – 7.52 (m, 1H), 7.14 – 7.01 (m, 2H), 6.83 (dd, *J* = 8.4, 2.5 Hz, 1H), 6.73 (d, *J* = 2.4 Hz, 1H), 4.00 (s, 2H), 2.45 (s, 3H), 2.37 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 155.76 (C), 145.82 (C), 134.52 (C), 133.37 (C), 129.60 (CH), 129.12 (C), 125.10 (C), 124.55 (CH), 124.31 (CH), 120.96 (CH), 114.66 (CH), 113.75 (CH), 55.23 (CH_2), 40.42 (CH_3), 17.59 (CH_3); HRESIMS *m/z* 226.1228 $[M+H]^+$ (calcd for $C_{15}H_{16}NO$, 226.1226).

5-ethyl-4-methyl-5,6-dihydrophenanthridine-8,9-diol (12). 81% yield; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45 (d, *J* = 7.7 Hz, 1H), 7.24 (s, 1H), 7.11 (d, *J* = 7.0 Hz, 1H), 7.05 (t, *J* = 7.6 Hz, 1H), 6.73 (s, 1H), 3.99 (s, 2H), 2.68 (q, *J* = 7.1 Hz, 2H), 2.34 (s,

3H), 1.07 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 143.44 (C), 142.85 (C), 141.90 (C), 133.42 (C), 129.84 (CH), 129.17 (C), 126.59 (C), 126.14 (C), 123.87 (CH), 120.86 (CH), 113.57 (CH), 110.43 (CH), 49.35 (CH_2), 45.93 (CH_2), 17.78 (CH_3), 13.58 (CH_3); HRESIMS *m/z* 256.1322 $[M+H]^+$ (calcd for $C_{16}H_{18}NO_2$, 256.1332).

Alkylation of Compounds 10, 11 and 12. . The lycorine derivatives **10**, **11** and **12** (0.1 mmol) were dissolved in dry THF (10 mL), and NaH (50 mg, 2 mmol) and 3-(bromomethyl)pyridine, 3-(Chloromethyl)-1,5-dimethyl-1H-pyrazole or 5-Bromomethyl-1,3-dimethyl-1H-pyrazole (1 mmol) were added. The mixture was stirred at r.t. for 24 h and quenched with H_2O (50 mL) in an ice bath. The solution was evaporated to remove the THF and extracted with CH_2Cl_2 (2 × 30 mL). The organic layer was washed with saturated $NaHCO_3$ and brine, dried over $MgSO_4$, filtered and concentrated. The residue was purified by column chromatography using petroleum ether-EtOAc as the eluent to afford compounds **5**, **6**, **7**, **8** and **9.**

8-((1,3-dimethyl-1H-pyrazol-5-yl)methoxy)-5-ethyl-4-methyl-5,6-dihydro-phenanthridine (5). 78% yield. Colorless amorphous powder (from CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.59 (d, *J* = 8.5 Hz, 1H), 7.50 (d, *J* = 7.6 Hz, 1H), 7.06 (d, *J* = 8.0 Hz, 1H), 7.00 (t, *J* = 7.5 Hz, 1H), 6.86 (dd, *J* = 8.5, 2.6 Hz, 1H), 6.74 (d, *J* = 2.5 Hz, 1H), 6.04 (s, 1H), 4.94 (s, 2H), 4.01 (s, 2H), 3.79 (s, 3H), 2.62 (q, *J* = 7.1 Hz, 2H), 2.29 (s, 3H), 2.19 (s, 3H), 1.01 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.77 (C), 147.36 (C), 146.31 (C), 137.89 (C), 135.20 (C), 133.43 (C), 129.94 (CH), 129.12 (C), 126.72 (C), 124.38 (CH), 123.89 (CH), 120.90 (CH), 113.66 (CH), 112.84 (CH), 106.77 (CH), 60.61 (CH_2), 50.18 (CH_2), 46.12 (CH_2), 36.50 (CH_3), 17.79 (CH_3), 13.63 (CH_3), 13.43 (CH_3); HRESIMS *m/z* 348.2055 $[M+H]^+$ (calcd for $C_{22}H_{26}N_3O$, 348.2070).

8,9-bis((1,3-dimethyl-1H-pyrazol-5-yl)methoxy)-5-ethyl-4-methyl-5,6-dihydro-phenanthridine (6). 75% yield. Colorless amorphous powder (from $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.49 (d, *J* = 7.2 Hz, 1H), 7.34 (s, 1H), 7.15 (d, *J* = 7.4 Hz, 1H), 7.08 (t, *J* = 7.6 Hz, 1H), 6.82 (s, 1H), 6.05 (s, 1H), 6.04 (s, 1H), 5.04 (s, 2H),

5.02 (s, 2H), 4.04 (s, 2H), 3.82 (s, 3H), 3.81 (s, 3H), 2.69 (q, *J* = 7.1 Hz, 2H), 2.36 (s, 3H), 2.24 (s, 6H), 1.08 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 148.26 (C), 147.91 (C), 147.23 (C), 146.43 (2C), 138.06 (C), 137.86 (C), 133.57 (C), 130.29 (CH), 128.86 (C), 127.92 (C), 127.38 (C), 123.86 (CH), 120.88 (CH), 114.04 (CH), 111.55 (CH), 106.90 (CH), 106.85 (CH), 62.56 (CH_2), 62.18 (CH_2), 49.53 (CH_2), 46.02 (CH_2), 36.37 (CH_3), 36.35 (CH_3), 17.80 (CH_3), 14.15 (CH_3), 13.64 (CH_3), 13.41 (CH_3); HRESIMS *m/z* 472.2688 $[M+H]^+$ (calcd for $C_{28}H_{34}N_5O_2$, 472.2707).

8-((1,5-dimethyl-1H-pyrazol-3-yl)methoxy)-5-ethyl-4-methyl-5,6-dihydro-phenanthridine (7). 23.9 mg, 87% yield. Colorless amorphous powder (from CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.63 (d, *J* = 8.5 Hz, 1H), 7.56 (d, *J* = 7.5 Hz, 1H), 7.11 (d, *J* = 7.3 Hz, 1H), 7.05 (t, *J* = 7.5 Hz, 1H), 6.97 (dd, *J* = 8.5, 2.6 Hz, 1H), 6.85 (d, *J* = 2.5 Hz, 1H), 6.13 (s, 1H), 5.04 (s, 2H), 4.06 (s, 2H), 3.78 (s, 3H), 2.66 (q, *J* = 7.1 Hz, 2H), 2.35 (s, 3H), 2.27 (s, 3H), 1.07 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 158.50 (C), 146.95 (C), 146.29 (C), 139.69 (C), 134.90 (C), 133.32 (C), 129.64 (CH), 129.41 (C), 125.96 (C), 124.22 (CH), 123.80 (CH), 120.82 (CH), 113.74 (CH), 112.87 (CH), 105.02 (CH), 64.19 (CH_2), 50.22 (CH_2), 46.06 (CH_2), 36.09 (CH_3), 17.79 (CH_3), 13.60 (CH_3), 11.28 (CH_3); HRESIMS *m/z* 348.2055 $[M+H]^+$ (calcd for $C_{22}H_{26}N_3O$, 348.2070).

5-ethyl-4-methyl-8-(pyridin-3-ylmethoxy)-5,6-dihydrophenanthridine (8). 85% yield. Colorless amorphous powder (from $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.62 (d, *J* = 4.2 Hz, 1H), 7.73 (td, *J* = 7.7, 1.7 Hz, 1H), 7.65 (d, *J* = 8.5 Hz, 1H), 7.56 (dd, *J* = 7.2, 4.0 Hz, 2H), 7.25 – 7.22 (m, 1H), 7.12 (d, *J* = 7.4 Hz, 1H), 7.06 (t, *J* = 7.5 Hz, 1H), 6.96 (dd, *J* = 8.5, 2.6 Hz, 1H), 6.86 (d, *J* = 2.5 Hz, 1H), 5.25 (s, 2H), 4.07 (s, 2H), 2.68 (q, *J* = 7.1 Hz, 2H), 2.35 (s, 3H), 1.07 (t, *J* = 7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 158.10 (C), 157.29 (C), 149.27 (CH), 146.31 (C), 136.89 (CH), 135.13 (C), 133.37 (C), 129.79 (CH), 129.25 (C), 126.36 (C), 124.36 (CH), 123.84 (CH), 122.68 (CH), 121.34 (CH), 120.86 (CH), 113.71 (CH), 112.96 (CH), 70.71 (CH_2), 50.20 (CH_2), 46.10 (CH_2), 17.79 (CH_3), 13.61 (CH_3); HREIMS *m/z* 330.1730 $[M]^+$ (calcd for $C_{22}H_{22}N_2O$, 330.1732).

5-ethyl-4-methyl-8,9-bis(pyridin-3-ylmethoxy)-5,6-dihydrophenanthridine (9). 76% yield. Colorless amorphous powder (from $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ 8.64 (s, 2H), 7.71 (t, J = 7.3 Hz, 2H), 7.63 (t, J = 8.9 Hz, 2H), 7.42 (d, J = 7.6 Hz, 1H), 7.34 (s, 1H), 7.23 (m, 2H), 7.10 (d, J = 7.4 Hz, 1H), 7.03 (t, J = 7.6 Hz, 1H), 6.80 (s, 1H), 5.39 (s, 2H), 5.36 (s, 2H), 3.98 (s, 2H), 2.64 (q, J = 7.1 Hz, 2H), 2.32 (s, 3H), 1.03 (t, J = 7.1 Hz, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 157.54 (C), 157.40 (C), 149.19 (2CH), 148.13 (C), 147.81 (C), 146.38 (C), 136.99 (2CH), 133.39 (C), 130.02 (CH), 129.03 (C), 127.09 (C), 126.60 (C), 123.79 (CH), 122.71 (2CH), 121.42 (2CH), 120.86 (CH), 112.81 (CH), 110.13 (CH), 72.21 (CH_2), 71.91 (CH_2), 49.50 (CH_2), 45.94 (CH_2), 17.78 (CH_3), 13.62 (CH_3); HRESIMS *m/z* 438.2176 $[M+H]^+$ (calcd for $C_{28}H_{28}N_3O_2$, 438.2180).

4.2 Bioactivity Assay

Cell Culture.

HEK293T cells were cultured i n Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated FBS in a humidified 5% CO_2/95% air (v/v) atmosphere at 37°C.

Reporter Gene Assay.

HEK293T cells were transfected using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. For reporter gene assays, HEK293T cells were seeded in 24-well plates. Each well was transfected with 250 ng of plasmids in total, including 20 ng of TOP Flash and 25 ng of EGFP-C1. The LacZ plasmid was added to equalize the total amount of plasmid in the wells to 250 ng. Eighteen hours after transfection, the cells were treated with Wnt3a-conditioned medium (Wnt3a CM) or control medium (Ctr CM) for an additional 6 h and were lysed using a Boehringer Mannheim Luciferase Assay Kit (200 μL/well) for luciferase assays. The fluorescence intensity emitted by green fluorescent protein (GFP) in the resultant cell lysates were first determined in a Wallac multi-counter capable of counting fluorescence and luminescence. Next, the luciferase substrate was added to the cell lysates, and the

luciferase activities were determined by measuring the luminescence intensities using the same counter. The luminescence intensities were normalized against the fluorescence intensities.

Axin (GenBank Accession No. NM_003502.3) were subcloned into pCMV vector to generate varying tagged fusion proteins. DAX domain of Axin and were subcloned into pET21a vector (Novagen, Darmstadt, Germany) to generate 6×His-tagged fusion proteins.

Target Gene Assay.

Cells were treated with the synthesized compounds as indicated and control or Wnt3a CM for 6 h. Total RNA was extracted with TRIzol. Additionally, purified RNA was reverse transcribed using oligo (dT) priming and the Superscript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. The gene transcripts were quantified by quantitative real-time PCR using a Quantitative SYBR green PCR kit (Takara SYBR premix Ex Taq) and ABI Quant Studio 6. Gene expression was normalized by GAPDH. The following primer pairs were used for the target genes: *Axin2*: 5'-AGGCTAGCTGAGGTGT -3' and 5'-AGGCTTGGATTGGAGAA-3'; *DKK1*: 5'-CTGCAAAAATGGAATATG-3' and 5'-CTTCTTGTCCTTTGGTGTGA-3'; *GAPDH*: 5'-AGGTCGGAGTCAACGGATT TG-3' and 5'-TGTAAACCATGTAGTTGAGGTCA-3'.

Compounds-Axin protein binding assay

Mouse fragments of Axin DIX domain were subcloned into pET32a vector to generate a His_6-taged fusion protein. Recombinant proteins were expressed and purified from Escherichia coli.The binding was incubation in PBS buffer contain 0.05% Triton X-100, 5mM beta-mercaptoethanol. The protein contains 80μM competing compounds or DMSO was incubated with Streptavidin Agarose (Invitrogen SA10004), which has been binded by 20 μM HLY179 molecular. After 1-2 hours of incubation, the beads were washed two timesand resuspended in 40 μL SDS loading buffer, and the bound proteins were analyzed by western blotting.

4.3 Molecular Docking Protocol

Compounds **12** and HLY78 were docked with EPAC2 using the X-ray structure of axin (PDB code: 1WSR) and AutoDock 4.2. The H_2O molecules and ligand within the crystal structure were removed, and polar hydrogen moieties were added using AutoDockTools. In the structures of the analogues, all bonds were rotatable except the aromatic, amide, cyano, and double bonds; the protein was treated as a rigid structure. The docking runs were performed using the standard parameters of the program for interactive growth and subsequent scoring.

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Supporting Information.

Supplementary data related to this article can be found at

Authors' Contributions

[1]These authors contributed equally.

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The manuscript was written through contributions of all authors. D-Z Chen and B-J Yang designed and performed the chemical synthesis under the guidance of X-J Hao; J-Y Cai, Y Zhang and J-J Guo purified all the compounds; X-L He, H Zhang, and Y-F Zhou evaluated the Wnt active activities and other bio-activities of all compounds

under the guidance of L Lin.; D-Z Chen and X-L He. wrote the manuscript with advice from all of the authors; X.H. guided all aspects of this study. All authors have given approval to the final version of the manuscript.

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Highlights

- Certain Wnt signalling agonists could be used to treat some Wnt-related diseases.
- HLY78 could become efficient Wnt agonists through appropriate modifications.
- Pyrazole substituents at C-8 or C-9 could improve Wnt activation of phenanthridines.
- The basic nitrogen at N-5 position of HLY78 are crucial to the Wnt active activity.