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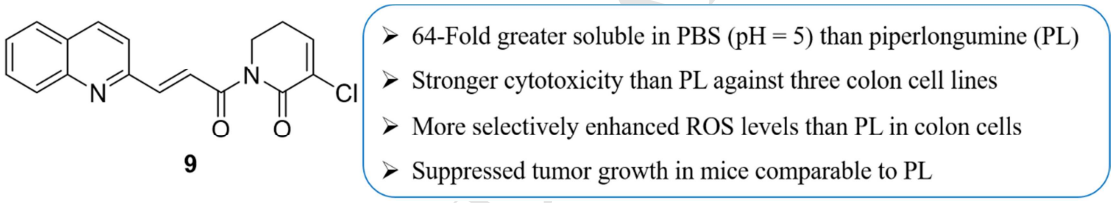
 # These authors contributed equally to this work.

Graphical Abstract



Synthesis and evaluation of N-heteroaromatic ring-based analogs of piperlongumine as potent anticancer agents

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Abstract

Piperlongumine (PL) selectively targets a wide spectrum of cancer cells and induces their death by triggering various pathways, including apoptosis, necrosis and autophagy. However, the poor solubility is a serious concern for intensive study and clinical application. We synthesized its analogs **1**−**9** by replacement of the trimethoxyphenyl of PL with an N-heteroaromatic ring and/or not introduction of 2-Cl. These compounds improved aqueous solubility and displayed potent anticancer activity. The most active compound **9** selectively enhanced ROS levels in colon cancer cells and inhibited the cell proliferation but sparing non-tumor colon cells. Importantly, **9** significantly repressed tumor growth in an HCT-116 xenograft mouse model, suggesting that these N-heteroaromatic ring-based analogs of PL warrant further investigation.

Keywords: piperlongumine, N-heteroaromatic ring, solubility, anticancer activity, ROS.

1. Introduction

Solubility is one of the key issues for drug candidates. Poor aqueous solubility often results in poor absorption, even though the permeation ability is great, since the flux of a drug cross the intestinal membrane is closely related to the concentration gradient between the intestinal lumen and blood. Additionally, efficacy and risk evaluation of poorly soluble compounds is challenging because exposure may be hard to define and the sensitivity of the assays may be diminished. Furthermore, high concentrations of poorly soluble drugs in some organisms may lead to crystallization and acute toxicity in cases such as uric acid and gout. As a matter of fact, the poor solubility has resulted in failures of numerous drug candidate development [1].

Appropriate formulation may be helpful in solving these problems [2], but the extent of solubility enhancement that can really be achieved is extremely limited. Additionally, stability and manufacturing problems also have to be in consideration, since an insoluble drug candidate may require a non or less conventional strategy, resulting in an increase in cost [3]. Therefore, it would be desirable to generate drug candidates with an applicable aqueous solubility at the early stage of drug development. To this regard, structural modification of the molecule is supposed to be much better than other approaches.

Piperlongumine (PL, Fig. 1) is a naturally occurring alkaloid isolated from the plant long pepper, and used traditionally for the treatment of certain diseases, such as antiplatelet aggregation [4], anti-inflammation [5], and cardiovascular protection [6]. PL has recently been shown to selectively target a wide spectrum of cancer cells [7] and induce cancer cell death by initiating various pathways, such as apoptosis, necrosis and autophagy [8,9]. The elevation of reactive oxygen species (ROS), characteristic of oxidative stress, is an important mechanism by which PL promotes cancer-selective cell death [7]. However, the poor aqueous solubility of PL is a serious concern for intensive investigations and clinical application [10,11]. Although a large variety of derivatives and analogs of PL have been synthesized in order to find agents with improved properties, few efforts are so far successful [12-15].

The analysis on the structure and biological activity relationship (SAR) of these

derivatives and analogs reveals that C2-C3 and C7-C8 double bonds are two pharmacophores as Michael acceptors essential for toxicity of PL to cancer cells (Fig. 1) [16]. The C2-C3 double bond is also critical for ROS elevation and glutathione depletion. 2-Halogenated PL possesses more potent in vitro anticancer activity than PL itself owing to its higher reactivity of C2-C3 Michael acceptor [17]. In addition, removal of C7-C8 olefin decreases cytotoxicity, but does not reduce the ROS levels [16]. Furthermore, it has been documented that the aromatic ring can be modified to obtain new analogs with better activity than PL [18].

It is well-known that N-heteroaromatics are generally more water soluble than corresponding aromatic compounds. For example, the aqueous solubility of pyridine and quinoline is 162.25 and 7.61 mg/mL [19], 92- and 224-fold greater than counterparts benzene (1.76 mg/mL) and naphthalene (0.034 mg/mL), respectively [20]. Accordingly, we hypothesized that replacement of the trimethoxyphenyl of PL by an N-heteroaromatic ring might improve the aqueous solubility and retain or increase the anticancer activity. In addition, given that 2-Cl substituted PL is more potent than PL [17], we therefore designed and synthesized new analogs **1-9** of PL (Fig. 1), and biologically evaluated their anticancer effects.

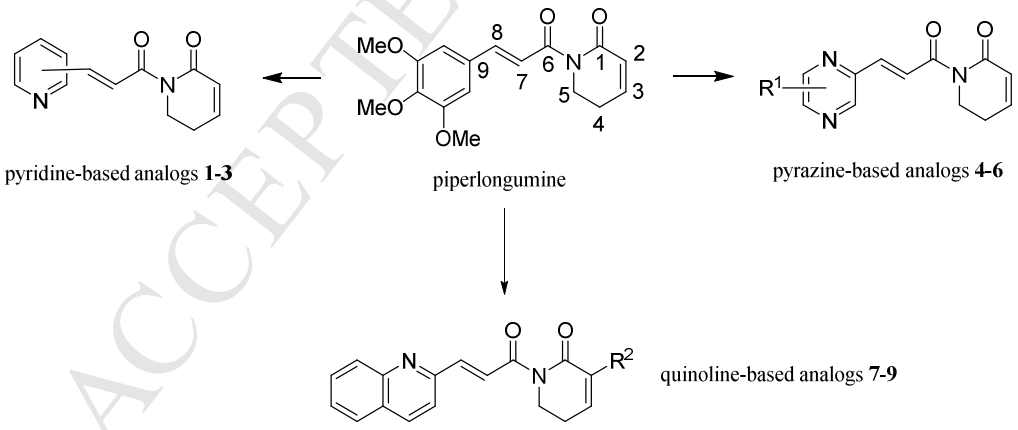


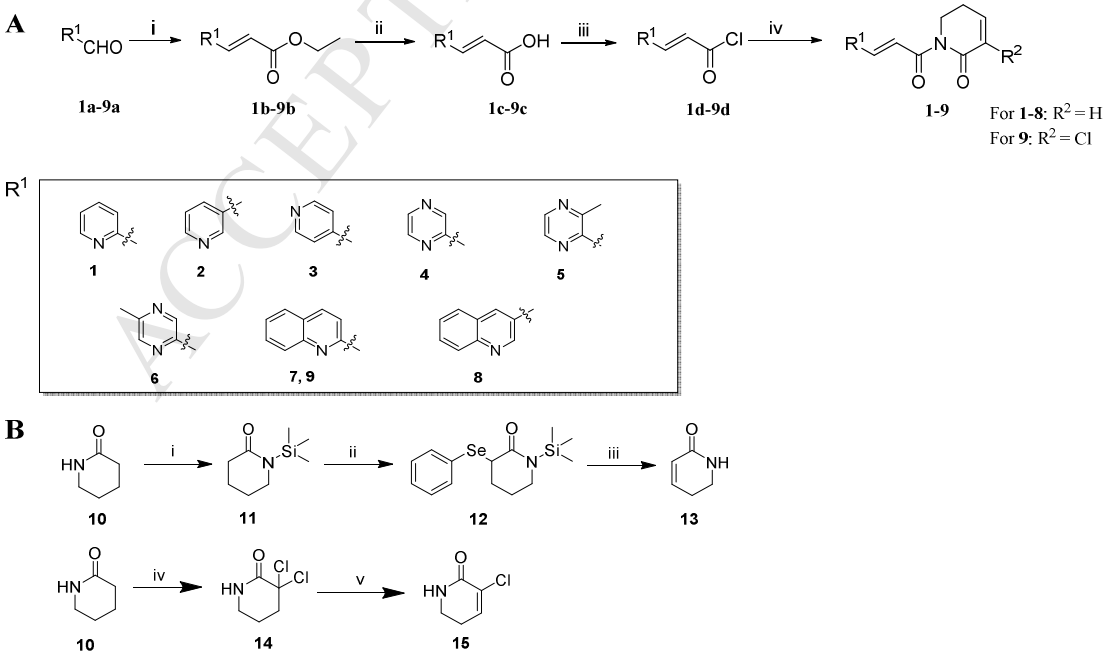
Fig. 1. Design of analogs **1-9** by replacement of the trimethoxyphenyl of PL with an N-heteroaromatic ring and/or not introduction of 2-Cl.

2. Results and discussion

2.1. Chemistry

The synthetic routes of target compounds **1-9** are shown in Scheme 1. Compounds **1a-9a** underwent Wittig-Horner reaction to generate ester **1b-9b**. Hydrolysis of **1b-9b** led to free acid **1c-9c**, which was converted to acylchloride **1d-9d**, without isolation followed by reacting with 5,6-dihydropyridin-2(*1H*)-one **13** and 6-chloro-5,6-dihydro-pyridin-2(*1H*)-one **15** to provide analogs **1-9**, respectively (Scheme 1A). Compound **13** was obtained by treatment of **10** with trimethyl chlorosilane (TMSCl), phenylselenyl chloride, and hydrogen peroxide, successively. Similarly, reaction of **10** with PCl_5 offered 2,2-dichloropiperidin-2-one **14**, followed by dehydrochlorination in the presence of Li_2CO_3 to provide 6-chloro-5,6-dihydropyridin-2(*1H*)-one **15** (Scheme 1B) [13].

The structures of all target compounds were fully characterized by ^{1}H-NMR, ^{13}C-NMR, MS, and HRMS, and the C7-C8 olefinic bond in these analogs was identified as E configuration according to the coupling constant of two corresponding protons on the double bond. The purity of the compounds was greater than 95%, determined by high performance liquid chromatography (HPLC) analysis, for the following biological experiments.



Scheme 1. A. The synthetic route of analogs **1-9**. Reagents and conditions: i.

Wittig-Hornor reaction; ii. KOH, 16 h; iii. anhydrous DCM, pivaloyl chloride, one drop of DMF; iv. anhydrous THF, TEA, n-butyllithium and compounds **13** or **15**. B. The synthetic route of compounds **13** and **15**. Reagents and conditions: i. toluene, TEA, TMSCl, 0 °C, 4 h; ii. THF, phenylselenyl chloride, LDA, -50 °C; iii. THF, 50%H_2O_2; iv. PCl_5, chloroform, r.t.; v. Li_2CO_3, LiCl, anhydrous DMF, 130 °C, 7 h.

2.2. Solubility evaluation

The solubility of PL and its analogs **1**-**8** was determined in pure water by HPLC [21] as shown in Table1. It was found that all analogs with solubility from 1.95 to 59.83 μg/mL were more soluble in the aqueous solution than PL (1.63 μg/mL). The effects of nitrogen-containing heteroaromatics are in the order of pyrzine > pyridine > quinoline. We reasoned that the improvement of the aqueous solubility of the analogs to various extent should be attributable to the different water-soluble N-heteroaromatics.

Table 1. The solubility (μg/mL) of compounds **1**-**8** and PL

compound	**1**	**2**	**3**	**4**	**5**
aqueous solubility	40.37	45.30	46.17	59.83	48.22
compound	**6**	**7**	**8**	PL	
aqueous solubility	47.91	2.41	1.95	1.63	

2.3. Assessment of in vitro anti-proliferative activity

Analogs **1**-**8** were first tested for their anti-proliferative activity against lung cancer A549, leukemic K562 and colorectal cancer HCT-116 cell lines by MTT assay. As shown in Fig. 2, all the analogs showed significant inhibitory activity comparable to PL. Among them, quinoline-based analog **7** was the most potent against HCT-116

cells. Since C2-chlorine substituted PL significantly enhanced the anticancer activity [17], we then introduced chlorine atom into C2 position of **7** in order to further improve its activity, yielding analog **9**, which had a similar solubility in pure water but an approximately 64-fold greater solubility in PBS aqueous solution (pH = 5) than PL (1992.7 *vs* 31.2 μg/ml). Subsequently, we determined IC_{50} values of compounds **1-9** against HCT-116 as shown in Table 2. Expectedly, **9** (0.47 ± 0.04 μM) exhibited the strongest cytotoxicity among all the tested compounds, superior to PL (8.13 ± 0.51 μM).

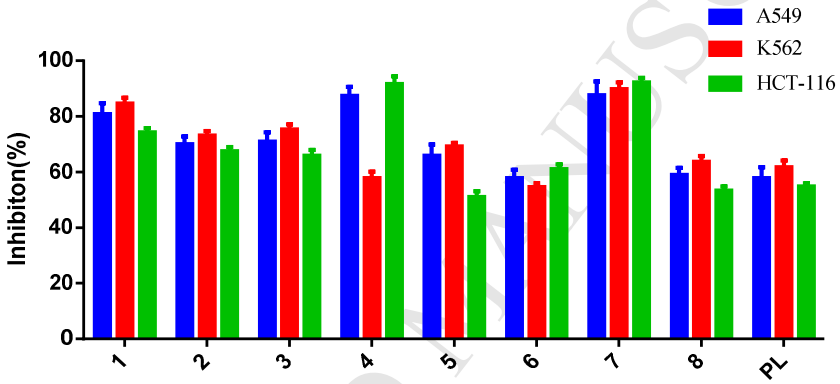


Fig. 2. Inhibitory activity of **1-8** at 10 μM against cancer A549, K562 and HCT-116 cells after incubation for 72 h. Cell proliferation was determined by MTT assay. Data are means ± SD of the inhibition (%) from three independent experiments.

Table 2. The IC_{50} (μM) of **1-9** and PL against HCT-116 cells

Compound	HCT-116	Compound	HCT-116
1	4.21 ± 0.26	**6**	7.95 ± 0.10
2	6.87 ± 0.31	**7**	0.93 ± 0.44
3	7.05 ± 0.24	**8**	8.17 ± 0.31
4	3.19 ± 0.12	**9**	0.47 ± 0.04
5	8.90 ± 0.17	PL	8.13 ± 0.51

Further tests indicated that **9** was also powerful against other two colon cancer cell

lines with IC_{50} values of 0.92 ± 0.11 μM and 1.79 ± 0.23 μM, respectively (Table 3). In sharp contrast, **9** showed much less inhibitory activity on non-tumor colon CDD-841 cells (IC_{50} = 50.81 ± 3.42 μM), better than PL (IC_{50} = 44.32 ± 1.77 μM) (Table 3), suggesting that **9** may selectively inhibit the colon cancer cells. Thus, **9** was selected for the following investigations.

Table 3. The IC_{50} (μM) of **9** against colon cancer cells and non-tumor colon cells [a]

Cell lines	HT-29	HCT-8	HCT-116	CCD-841
9	0.92 ± 0.11	1.79 ± 0.23	0.47 ± 0.04	50.81 ± 3.42
PL	2.65 ± 0.17	4.10 ± 0.20	8.13 ± 0.11	44.32 ± 1.77

[a] Cells were treated in triplicate with tested compounds for 72 h and the cell viability was determined by MTT assay.

2.4. ROS evaluations

Based on the above exciting results, we examined whether **9** would impact ROS levels in both colon cancer HCT-116 cells and non-tumor colon CCD-841 cells. HCT-116 and CCD-841 cells were treated with **9** and PL at 10 μM for 3 h, respectively, and the levels of intracellular ROS were detected by DCFH-DA probes using a fluorescent microplate reader to measure the fluorescent signals as shown in Fig. 3. It is obvious that treatment with **9** rapidly and significantly increased the levels of intracellular ROS in HCT-116 cells while the same treatment only increased low levels of ROS in CCD-841 cells. The selectivity of **9** was greater than that of PL, indicating that **9** more preferably promoted intracellular ROS accumulation relative to PL in the cancer cells.

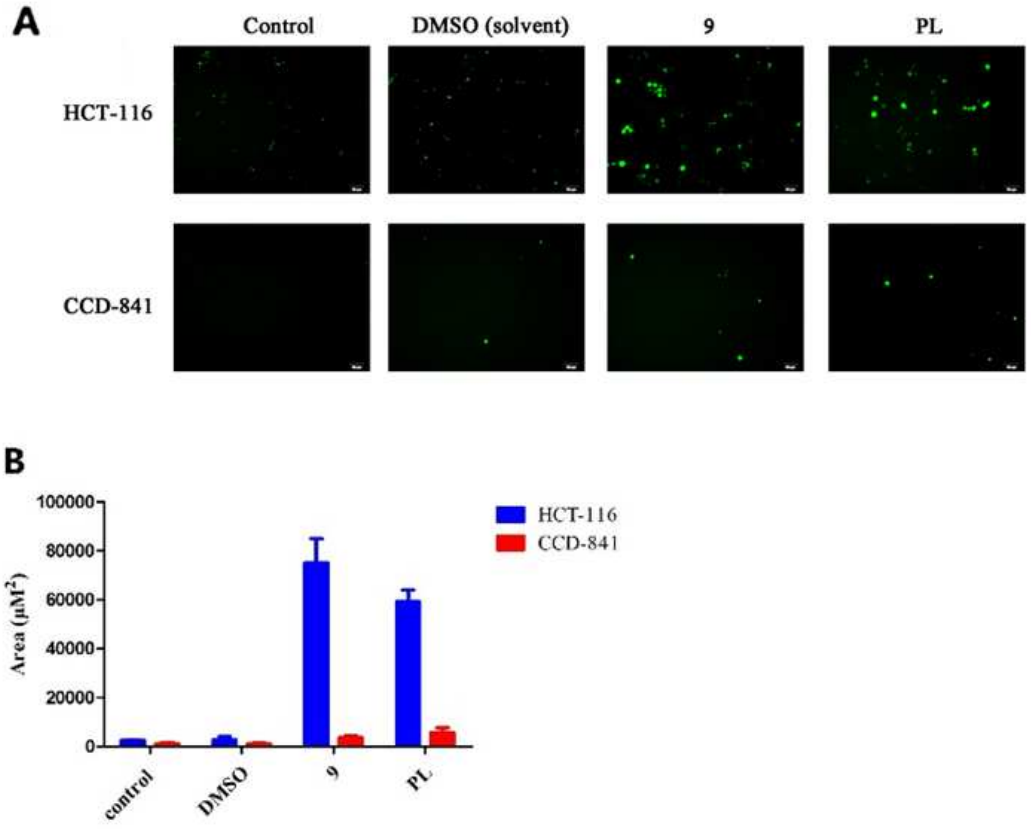


Fig. 3 . A. The fluorescence microscopy images of HCT-116 or CCD-841 cells treated with 10 μM PL or **9** for 3 h. B. The fluorescence intensities of the cells. HCT-116 or CCD-841 cell was treated with 10 μM PL or **9** for 3 h and then detected by fluorescence microscopy.

*2.5. In vivo anti-tumor effects of **9***

To evaluate the *in vivo* anti-colon cancer activity of **9**, BALB/c nude mice were inoculated subcutaneously with colon cancer HCT-116 cells. After the establishment of solid tumor, two groups of the mice were intraperitoneally treated with 5 mg/kg of **9** and PL daily for 21 consecutive days, respectively (Fig. 4). It could be seen from Fig. 4A and 4D that in comparison with the vehicle-treated controls, treatment with **9** at 5 mg/kg significantly inhibited the growth of implanted colon cancer cells (2.01 ± 0.34 vs 0.96 ± 0.19 g, $p < 0.01$), and the tumor weights in the mice were significantly reduced by 52 %, which was comparable to PL (53 %, w/w).

In addition, treatment with **9** significantly reduced the volumes and size of colon

tumors in mice (Fig. 4C). Moreover, there was no obvious abnormality in the liver, kidney, lung and heart in terms of the size and morphology in both control and **9**-treated mice except for a slight, but not significant reduction in body weight as compared with each other (Fig. 4B).

The reason why **9** showed better inhibitory activity *in vitro* but similar antitumor effects *in vivo* relative to PL is that **9** may have different targets in cancer cells or may not act on those affected by PL. However, the exact mechanism of action of **9** needs to be further clarified in future investigations.

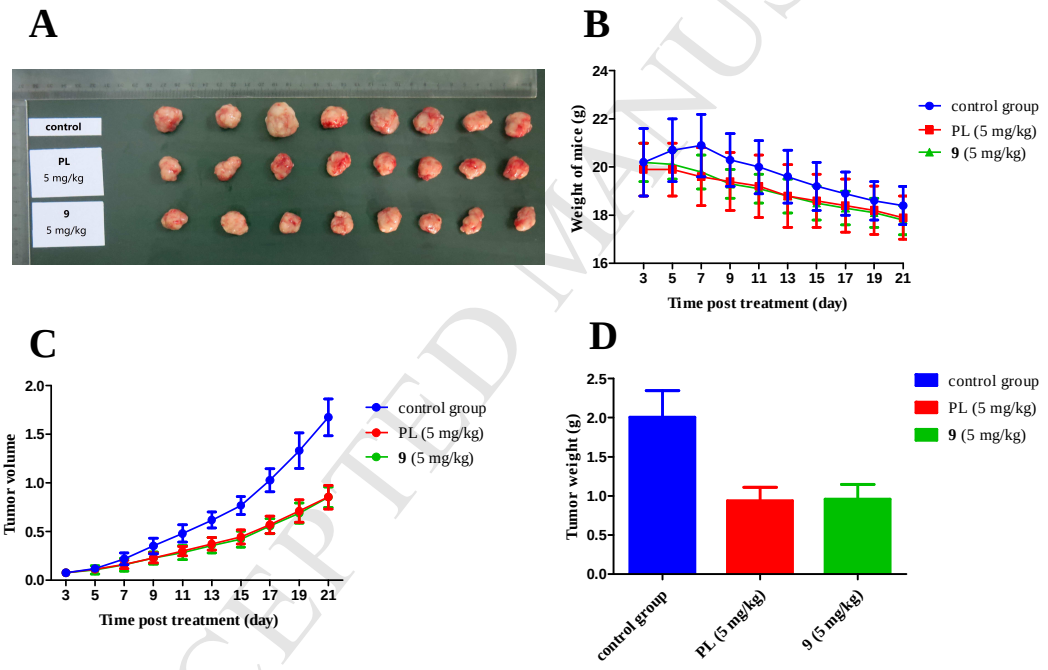


Fig. 4. Inhibitory effects of **9** on the growth of HCT-116 tumors *in vivo*. Mice inoculated with HCT-116 tumors were intraperitoneally treated with PL or **9** per day. A. Representative images of the tumors were captured. B. The weights of mice were measured at the indicated time points. C. The volume of tumors was measured at the indicated time points. D. The weight of tumors was measured at the indicated time points. Data are shown as means ± SD from each group of mice (n = 8). $^{**}P < 0.01$ vs control.

3. Conclusion

In summary, a series of analogs **1-9** were designed and synthesized by replacement of the trimethoxyphenyl of PL with an N-heteroaromatic ring and/or not introduction of 2-Cl. These compounds improved aqueous solubility and showed potent anticancer activity. The most active compound **9** selectively enhanced ROS levels and inhibited proliferation in colon cancer HCT-116 cells but sparing the non-tumor colon CCD-841 cells. Furthermore, **9** significantly inhibited the growth of implanted colon cancer cells in mice. Collectively, our findings suggest that these N-heteroaromatic ring-based analogs of PL may deserve intensive investigations.

4. Experimental protocols

4.1. General informations

Nuclear magnetic resonance (NMR) spectra were obtained from a Bruker Avance 300 (^{1}H, 300 MHz; ^{13}C, 75 MHz) or 500 (^{1}H, 500 MHz; ^{13}C, 125 MHz) spectrometer at 300 K using TMS as an internal standard. Mass spectrometry (MS) spectra were recorded on a Mariner mass spectrometer. Melting points (mp) were measured by a Mel-TEMP II apparatus and uncorrected. TLC was performed on silica gel GF/UV 254, and column chromatography was conducted by silica gel (200−300 mesh). The purities of target compounds were characterized by HPLC analysis (Shimadzu DGU-20A3R) and HRMS (Agilent Technologies LC/MSD TOF). The target compounds **1-9** with a purity of >95% were used for subsequent experiments. Compound **11-15** were prepared as described in reference 13 starting from commercially available compound **10**, and the synthetic procedures of compounds **11-15** were shown on pages S2 and S3 in the Supporting Information.

*4.1.1. General procedure for the preparation of **1-9***

Compound **1a-9a** (1.0 mmol) was dissolved in toluene (70 mL) in a flask which

was wrapped with tin foil, sodium hydride (1.5 mmol) and triethyl phosphonoacetate (1.0 mmol) was added respectively. Then the reaction solution was stirred at room temperature under dark until TLC analysis showed complete conversion. Extracted with EtOAc, the combined organic phase was washed with saturated brine (50 mL × 3), and dried over anhydrous sodium sulfate. Concentrated and purified by column chromatography (PE / EA = 8: 1) to give a pale yellow solid (**1b**-**9b**). The synthetic routes are similar to PL. [7, 12]

Compound **1b**-**9b** (1.0 mmol) was dissolved in a mixed solution of tetrahydrofuran and water (V(THF) / V(H_2O) = 2: 1, 54 mL), then lithium hydroxide (4.0 mmol) was slowly added, and the mixture was stirred overnight at room temperature, checking for product formation via TLC (PE / EA = 1: 1). Next, the pH value of reaction solution was adjusted to 4-5 with 2N hydrochloric acid aqueous solution. The mixture was then extracted with EtOAc (30 mL × 3), and the combined organic phase was washed with saturated brine (50 mL × 3), dried over anhydrous sodium sulfate and concentrated to give a pale yellow solid **1c**-**9c**. The synthetic routes are similar to PL. [7, 12]

Compound **1c**-**9c** (1.0 mmol) was dissolved in anhydrous tetrahydrofuran (20 mL). To the solution, triethylamine (1.2 mmol) was slowly added and the solution was cooled to -20 °C, pivaloyl chloride (1.2 mmol) was then added, and the reaction mixture was stirred for about 45 minutes. Next, the compound **13** or **15** and n-butyllithium were added to the reaction solution and stirred for 1 h, checked for product formation via TLC. Then saturated aqueous solution of ammonium chloride (10 mL) was added to quench the n-butyllithium in the reaction and extracted with ethyl acetate, the organic phase was washed with saturated brine (30 mL × 3), Dried over anhydrous sodium sulfate, concentrated and purified by column chromatography (PE / EA = 3: 1) to afford **1**-**9**.

*4.1.2.1 (E)-1-(3-(Pyridin-2-yl)acryloyl)-5,6-dihydropyridin-2(1H)-one (**1**).* Mp: 77-80 □; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.61 (s, 1H), 7.83-7.65 (m, 3H), 7.46 (d, *J* = 9.0 Hz, 1H), 7.16-7.11 (m, 1H), 6.94-6.91 (m, 1H), 6.02 (d, *J* = 9.0 Hz, 1H), 4.08-4.00

(m, 2H), 2.46 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.0, 165.8, 153.9, 150.3, 145.8, 142.0, 136.7, 126.1, 125.8, 124.1, 41.8, 25.0; HRMS (ESI +) *m/z*: Calcd. for $C_{13}H_{12}N_2O_2Na$ $[M+Na]^+$ 251. 0796, found 251.0801, ppm error 0.5.

*4.1.2.2 (E)-1-(3-(Pyridin-3-yl)acryloyl)-5,6-dihydropyridin-2(1H)-one (**2**).* Mp: 99-102 □; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.74 (s, 1H), 8.55 (s, 1H), 7.92-7.80 (m, 1H), 7.65-7.49 (m, 2H), 7.49-7.40 (m, 1H), 6.93 (s, 1H), 6.02 (d, *J* = 9.0 Hz, 1H), 4.02 (t, *J* = 6 Hz, 2H), 2.46 (s, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 168.2, 165.7, 153.8, 148.2, 145.7, 141.9, 136.7, 126.1, 125.8, 124.0, 41.8, 24.9; HRMS (ESI +) *m/z*: Calcd. for $C_{13}H_{12}N_2O_2Na$ $[M+Na]^+$ 251.0796, found 251.0803, ppm error 0.7.

*4.1.2.3 (E)-1-(3-(Pyridin-4-yl)acryloyl)-5,6-dihydropyridin-2(1H)-one (**3**).* Mp: 90-94 □; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.59 (s, 2H), 7.74-7.37 (m, 4H), 6.94 (d, *J* = 6 Hz, 1H), 6.02 (d, *J* = 9.0 Hz, 1H), 4.08-4.00 (m, 2H), 2.56-2.34 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ169.0, 164.7, 153.2, 148.0, 145.7, 141.9, 136.5, 126.1, 125.2, 124.1, 41.0, 24.9; HRMS (ESI +) *m/z*: Calcd. for $C_{13}H_{12}N_2O_2Na$ $[M+Na]^+$ 251.0796, found 251.0802, ppm error 0.6.

*4.1.2.4 (E)-1-(3-(Pyrazin-2-yl)acryloyl)-5,6-dihydropyridin-2(1H)-one (**4**).* Mp: 110-115 □; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.67 (s, 1H), 8.58 (s, 1H), 8.48 (s, 1H), 7.90 (d, *J* = 15.0 Hz, 1H), 7.63 (d, *J* = 15.0 Hz, 1H), 6.97-6.92 (m, 1H), 6.02 (d, *J* = 9.0 Hz, 1H), 4.02 (t, *J* = 12.0 Hz, 2H), 2.50-2.45 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 168.5, 149.5, 146.1, 145.1, 145.0, 144.9, 137.7, 131.1, 128.4, 125.7, 41.8, 25.0; HRMS (ESI +) *m/z*: Calcd. for $C_{12}H_{11}N_3O_2Na$ $[M+Na]^+$ 252.0749, found 252.0753, ppm error 0.4.

*4.1.2.5 (E)-1-(3-(3-Methylpyrazin-2-yl)acryloyl)-5,6-dihydropyridin-2(1H)-one (**5**).* Mp: 121-133 □; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.39 (d, *J* = 9.0 Hz, 2H), 7.94-7.79 (m, 2H), 6.96-6.90 (m, 1H), 6.02 (d, *J* = 12.0 Hz, 1H), 4.02 (t, *J* = 6.0 Hz, 2H), 2.67 (s, 3H), 2.49-2.44 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 168.8, 165.7, 153.1, 147.5,

145.9, 144.1, 142.5, 136.2, 128.9, 125.7, 41.8, 27.8, 21.8; HRMS (ESI +) *m/z*: Calcd. for $C_{13}H_{13}N_3O_2Na$ $[M+Na]^+$ 266.0905, found 266.0911, ppm error 0.6.

*4.1.2.6 (E)-1-(3-(5-Methylpyrazin-2-yl)acryloyl)-5,6-dihydropyridin-2(1H)-one (**6**)*. Mp: 175-180 □; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.55 (s, 1H), 8.45 (s, 1H), 7.85 (d, *J* = 15.0 Hz, 1H), 7.65 (d, *J* = 15.0 Hz, 1H), 6.97-6.91 (m, 1H), 6.03 (d, *J* = 12.0 Hz, 1H), 4.02 (t, *J* = 6.0 Hz, 2H), 2.57 (s, 3H), 2.50-2.45 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 168.8, 165.6, 157.5, 154.2, 146.7, 144.6, 142.3, 136.2, 128.1, 125.6, 41.8, 27.4, 20.9; HRMS (ESI +) *m/z*: Calcd. for $C_{13}H_{13}N_3O_2Na$ $[M+Na]^+$ 266.0905, found 266.0911, ppm error 0.6.

*4.1.2.7 (E)-1-(3-(Quinolin-2-yl)acryloyl)-5,6-dihydropyridin-2(1H)-one (**7**)*. Mp: 84-89 □; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.15-8.13 (m, 2H), 7.91 (s, 2H), 7.81 (d, *J* = 9.0 Hz, 1H), 7.69-7.66 (m, 2H), 7.55-7.50 (m, 1H), 6.98-6.92 (m, 1H), 6.05 (d, *J* = 9.0 Hz, 1H), 4.05 (t, *J* = 6.0 Hz, 2H), 2.54-2.46 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ168.4, 162.8, 153.9, 148.5, 146.7, 143.9, 141.5, 130.3, 130.1, 128.3, 127.4, 126.6, 126.2, 120.4, 41.9, 27.8; HRMS (ESI +) *m/z*: Calcd. for $C_{17}H_{14}N_2O_2Na$ $[M+Na]^+$ 301.0953, found 301.0960, ppm error 0.7.

*4.1.2.8 (E)-1-(3-(Quinoline-3-yl)acryloyl)-5,6-dihydropyridin-2(1H)-one (**8**)*. Mp: 86-91 □; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.48 (s, 1H), 8.12 (dd, *J* = 15.0 and 6.0 Hz, 2H), 7.93 (s, 2H), 7.77 (d, *J* = 15.0 Hz, 1H), 7.69-7.72 (m, 2H), 7.54 (t, *J* = 7.5 Hz, 1H), 6.07 (d, *J* = 9.0 Hz, 1H), 4.07 (t, *J* = 6.0 Hz, 2H), 2.52-2.45 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 168.4, 162.8, 149.3, 148.5, 146.7, 143.9, 141.5, 130.1, 129.7, 128.3, 127.6, 127.4, 127.0, 126.2, 41.9, 27.8; HRMS (ESI +) *m/z*: Calcd. for $C_{17}H_{14}N_2O_2Na$ $[M+Na]^+$ 301.0953, found 301.0960, ppm error 0.7.

*4.1.2.9 (E)-3-Chloro-1-(3-(quinoline-2-yl)acryloyl)-5,6-dihydropyridin-2(1H)-one (**9**)*. Mp: 93-96 □; ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.08 (dd, *J* = 15.0 and 6.0 Hz, 2H), 7.91-7.76 (m, 2H), 7.74 (d, *J* = 12.0 Hz, 1H), 7.70-7.67 (m, 2H), 7.52 (t, *J* = 6.0 Hz),

7.10-7.03 (m, 1H), 4.09 (t, *J* = 6.0 Hz, 2H), 2.56 (d, *J* = 6.0 Hz, 2H) ; ^{13}C NMR (75 MHz, $CDCl_3$): δ 168.4, 161.6, 153.9, 148.5, 143.9, 141.5, 136.8, 130.2, 130.1, 127.7, 127.5, 127.4, 126.6, 120.5, 42.0, 25.5; HRMS (ESI +) *m/z*: Calcd. for $C_{17}H_{13}ClN_2O_2Na$ $[M+Na]^+$ 335.0563, found 336.0571, ppm error 0.8.

4.2 Solubility test

Compounds **1**-**8** and PL were added to the indicated solvent. After shaking and centrifuging, the supernatant was taken to determine the concentration of the compounds for calculation of the corresponding solubility. The samples (20 μL each) were analyzed by HPLC (Shimadzu DGU-20A3R) on Shimadzu-GL WondaSil C18-WR column. The mobile phase was acetonitrile–water (60/40, v/v) at a flow rate of 1.0 mL/min with the detection wavelength at 254 nm. This experiment was repeated in triplicates.

4.3 Biological assays

4.3.1 MTT assay

Human colon, colorectal and non-tumor colon epithelial cells (1×10^6 cells/well) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in 96-well plates overnight and treated with vehicle alone or the tested compounds at the indicated concentrations for 24, 48, or 72 h. During the last 4-h culture, the cells were exposed to 20 μL of MTT (5 mg/mL, in PBS). The generated formazan in individual wells was dissolved in DMSO (150 μL), and measured for the absorbance at 570 nm using a microplate reader. The inhibition rates were calculated by the formula of (1-OD (experimental cells)/OD (control cells)) x 100%.

4.3.2 Measurement of ROS generation

The tested cells (1×10^6 cells/well) were treated in triplicate with vehicle DMSO (0.1%, v/v) alone, PL or **9** at the indicated concentrations for 3 h, and the cells were stained with dihydroethidium (DHE, Beyotime). The levels of intracellular ROS were measured as the fluorescent signals using a fluorescence microplate reader (300 and

610 nm).

4.3.3 Evaluation of in vivo antitumor activity

All animal experimental protocols were approved by the Administration Committee of Experimental Animals in Jiangsu Province and the Ethics Committee of China Pharmaceutical University. Male BALB/c nude mice 3–5 weeks of age (Silaike Experiment Animal Co., Ltd, Shanghai, China) were inoculated subcutaneously with 1×10^7 HCT-116 cells. After the formation of a solid tumor with a volume of about 75 mm^3, the tumor-bearing mice were randomly divided into four groups with eight mice in each group. The groups with compound **9** and PL treatment received a dosage (5 mg/kg) every day. At the end of the experiment, the mice were sacrificed, their tumors were dissected, and the tumor size and weight were measured. The tumor inhibitory ratio was calculated by the following formula: tumor inhibitory ratio (%) = [(Wcontrol − Wtreated)/Wcontrol] × 100%. $W_{treated}$ and $W_{control}$ were the average tumor weights of the treated and control mice, respectively. The tumor diameters were measured with calipers, and the tumor volume was calculated by the formula V (mm^3) = d2 × D/^{2}, where D is the largest diameter and d the smallest diameter.

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Highlights

- **9** was 64-fold greater soluble in PBS aqueous solution (pH = 5) than piperlongumine (PL).
- **9** was more cytotoxic (IC_{50} = 0.47-1.79 μM) than PL (IC_{50} = 2.65-8.13 μM) against three colon cancer cell lines but sparing non-tumor colon cells.
- **9** more preferably promoted ROS accumulation than PL in colon cells.
- **9** significantly suppressed tumor growth in vivo comparable to PL.