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Graphic Abstract

R₁: Alkyl is preferred
R₂: Halogen is preferred;
Morpholine is inactive
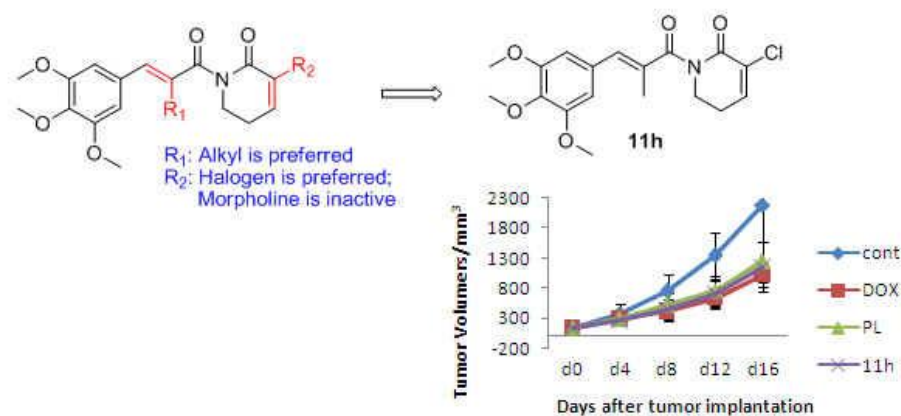
Sixteen piperlongumine derivatives with halogen or morpholine at C2 and alkyl substituents at C7 were prepared as anticancer agents. The highly active anticancer compound **11h** exhibited obvious ROS elevation and excellent *in vivo* antitumor potency.

Highlights

◆   Sixteen piperlongumine derivatives were prepared in five or six steps.

◆   2-Halogen piperlongumines showed moderate *in vitro* activity against four cancer cells.

◆   Most of the compounds showed modest selectivity for lung normal cells.

◆   Halogen substituents at C2 played an important role in increasing cytotoxicity.

Design, Synthesis and biological activity of piperlongumine derivatives as selective anticancer agents

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Abstract: In an effort to expand the structure-activity relationship of the natural anticancer compound piperlongumine, we have prepared sixteen novel piperlongumine derivatives with halogen or morpholine substituents at C2 and alkyl substituents at C7. Most of 2-halogenated piperlongumines showed potent *in vitro* activity against four cancer cells and modest selectivity for lung normal cells. The highly active anticancer compound **11h** exhibited obvious ROS elevation and excellent *in vivo* antitumor potency with suppressed tumor growth by 48.58% at the dose of 2 mg/ kg. The results indicated that halogen substituents as electrophilic group at C2 played an important role in increasing cytotoxicity.

1. Introduction

Piperlongumine(PL) is an alkaloid isolated from *Piper* species and displays anticancer[1-7], anti-diabetic[8], anti-platelet aggregation[9-12], and antifungal activities[13, 14]. Recently, Raj *et al* demonstrated selectivity of piperlongumine for cancer cells. Compared to the normal cell lines, piperlongumine induced cell death in thirteen cancer lines with an average IC_{50} value lower than 7 μM[15]. Additionally, similar effects were observed in vivo in four nude mice models without effect on normal tissue. Further studies revealed a unique mechanism based on reactive oxygen species (ROS). Using a phenotypic screen, they found that two proteins: glutathione-S- transferase-P1 (GSTP1) and carbonyl reductase 1 (CBR1) played a more important role in the cellular response to ROS. This tumor specific effect of piperlongumine on cytotoxicity and ROS shows a new strategy for selective targeting of cancer cells[16-20].

To explore the structure and biological activity relationship of piperlongumine, eighty analogues had been synthesized and screened by Adams *et al*[21]. Based on the assays of cellular toxicities, two key pharmacophores: C2-C3 and C7-C8 double bonds had been identified. Both these Michael acceptors were necessary for PL's toxicity to cancer cells. The former was also critical for ROS elevation and glutathione depletion. However, removal of C7-C8 olefin led to a decrease in cytotoxicity, but did not reduce the ROS level. Among all the analogues, PL dimer and trimer displayed more potent activities than the others. SAR indicated that alkynyl substituents can be introduced at position 2 without effect in cytotoxicity. On the contrary, alkyl and aromatic

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substituents were not tolerated. Although the C7-C8 olefin has been confirmed as one

of two key pharmacophores for cellular toxicity with the example of the inactive

C7-C8 olefin reduction product, we are unaware of the effect of substituents on the

C7-C8 double bond. In order to continue the development of piperlongumine

derivatives as selective anticancer agents, we describe herein the synthesis and

biological activity of a novel series of piperlongumine derivatives on positions 2 and

7. An active piperlongumine derivative was also assayed for its *in vivo* antitumor

activity in aA549 nude mice model.

2. Chemistry

PL and sixteen derivatives were synthesized according to the modified Raj's route[15].

As key reaction intermediates, three dihydropyridin-2(1*H*)-ones **3**, **4** and **6** were

prepared in three or four steps from the commercially available piperidin-2-one

1(Scheme 1). Treatment of **1** with PCl_5 in chloroform yielded

2,2-dichloropiperidin-2-one **2**[22, 23]. Dehydrochloration of **2** at 120°C in the

presence of Li_2CO_3 provided the corresponding 2-chloro- 5,6-dihydropyridin-

2(1*H*)-one **3** which was readily converted into 2-morpholino- 5,6-dihydropyridin-

2(1*H*)-one **4**. The third dihydropyridin-2(1*H*)-one **6** was prepared by the same route as

3 in a 63.1% yield.

With three dihydropyridin-2(1*H*)-ones in hand, all target piperlongumine derivatives

were synthesized as detailed in Scheme 2. Condensation of 3,4,5-trimethoxy-

benzaldehyde **7** with 2-substituted malonic acids in pyridine yielded 2-substituted

(*E*)-3-(3,4,5-trimethoxyphenyl)acrylic acids **9a** - **9e**. Treatment of **9a** - **9e** with oxalyl

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chloride in anhydrous THF under N_2 gave the acyl chlorides **10a** - **10e** which were reacted with the three substituted dihydropyridin-2(1*H*)-ones to afford piperlongumine derivatives **11a** − **11p** using anhydrous THF as solvent and TEA as catalyst.

3. Results and discussion

3.1 *In Vitro* anticancer screening

The first obtained seven piperlongumine derivatives **11a**-**11g** and PL were evaluated for *in vitro* cytotoxicities against four cancer cells (human lung carcinoma A549, human colorectal carcinoma HCT116, human breast carcinoma ZR-75-30, human breast carcinoma MDA-MB-231) and human lung normal cells MRC-5 by MTT assay, respectively. A summary of these results is shown in Table 1. Interestingly, substituents on position 2 of piperlongumine played an important role in antiproliferative activities. For example, compounds **11a** and **11b** without any group at C2 exhibited potent activities. On the other hand, the 2-chloro derivative **11g** also showed potent activities against all four cells. However, compounds **11c** - **11f** with morpholine substituents at C2 were uniformly less active than piperlongumine. In addition, compound **11g** resulted in a nearly two- or three-fold increase in selectivity for three cancer cell lines HCT116, ZR-75-30, MDA-MB-231 over normal cells MRC-5.

Encouraged by the preliminary design, a series of piperlongumine derivatives with halogen substituent at C2 were synthesized to improve antitumor activity and investigate the SARs. To our delight, most of the 2-halogen substituted

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piperlongumine derivatives showed potent activities against four cancer cell lines (human lung carcinoma A549, human colorectal carcinoma HCT116, human breast carcinoma MDA-MB-231, human hepatoma Hep3B) and human fetal lung normal cells WI38 (**Table 2**). Remarkably, both 2-chloro substituted piperlongumines **11h** – **11k** and 2-bromo substituted piperlongumines **11l** – **11p** showed more potent activities than piperlongumine. This fact confirmed that halogen substituents as electrophilic group at C2 increase cytotoxicity. Moreover, selectivity for A549 cells over normal cells was slightly decreased with the length of carbon chain at C7 except for compound **11l**. For example, 7-methyl-2-chloropiperlongumine **11h** with a rate value of 4.98 showed higher selectivity than 7-propyl-2-chloropiperlongumine **11j** in these assays.

3.2 ROS evaluation

Although the natural product piperlongume, isolated from *Piper* species has been recognized as an antitumor agent, its cellular mechanism has not been defined. Recently a ROS-independent cellular effect has been disclosed as the antitumor mechanism of piperlongumine[15, 21]. In order to validate the mechanism of the target compounds, we next determined the effect of piperlongumine derivative **11h** on cellular ROS level in A549 cancer cells by fluorescence microscopy. Treatment with 10 μM of **11h** for 1h caused an obvious increase in ROS level (Fig. 2). The result indicated that a halogen substituent at C2 of piperlongumine did not affect the ROS elevation.

3.3 *In vivo* anticancer evaluation

On the basis of the favorable *in vitro* cellular activity and selectivity for normal cells, compound **11h** was selected for further *in vivo* antitumor activity studies on human lung cancer A549 xenograft model. Doxorubicin (DOX), a clinical drug in the treatment of a wide range of cancers, was chosen as reference. PL was also used as reference for the effect of the halogen substituent of piperlongumine on inhibition tumor growth. As showed in Fig. 3, the results revealed that remarkable antitumor effects were observed in mice treated with compound **11h** which was administered intraperitoneally (ip) for fourteen consecutive days. Furthermore, compound **11h** showed higher *in vivo* antitumor potency than natural piperlongumine.

At the dose of 2 mg/ kg body weight, compound **11h** suppressed tumor growth by 48.58% compared to 38.31% of PL at the same dose. In addition, there is no obvious weight loss in animals treated with compound **11h**. Therefore, the results may suggest a low toxicity for compound **11h** which is worth of further studies as a new potential antitumor candidate.

4. Conclusions

In this work, a novel series of piperlongumine derivatives with halogen, morpholine substituents at C2 and alkyl substituents at C7 were synthesized and characterized. 2-Halo-7-alkylpiperlongumines exhibited moderate *in vitro* activity against all four cancer cells while the morpholine substituted derivatives at position 7 of piperlongumine showed diminished toxicities in cells. It was noted that all of the active compounds revealed modest selectivity for human lung normal cells MRC-5 and human fetal lung normal cells WI38. The highly active anticancer compound **11h**

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with selectivity for normal cells exhibited obvious ROS elevation and excellent *in vivo* antitumor potency. The present investigation indicated that 2-chloro-7-methyl-piperlongumine **11h** is worthy further of studies as an antitumor candidate.

5. Experimental Section

5.1 Chemistry. General Methods. All starting materials were commercially available and analytically pure. Melting points were measured on an uncorrected XT4A digital melting point apparatus (Beijing Keyi Instrument Co., Ltd.; China). ^{1}H NMR spectra were recorded on a Bruker Avance 300, Bruker Avance 500 and a Bruker Avance 600 spectrometers (Bruker Company, Germany), using TMS as an internal standard and $CDCl_3$ or DMSO-d_6 as solvents. Chemical shifts (δ values) and coupling constants (J values) are given in ppm and Hz, respectively. The mass spectra were recorded on an Esquire 3000 LC-MS mass spectrometer. TLC analysis was carried out on silica gel plates GF254 (Qindao Haiyang Chemical, China). Flash column chromatography was carried out on silica gel 300 - 400 mesh using a Biotage instrument. Anhydrous solvents and reagents were all analytically pure and dried by routine protocols.

5.2 3-Chloro-5,6-dihydropyridin-2(1*H*)-one (3)

A solution of piperidin-2-one (25.0 g, 252.2 mmol) in CH_3Cl (250 mL) was treated with PCl_5(158.0 g, 765.5 mmol) at 0-5 °C for 15 min before being refluxed for 3 h. Then the reaction mixture was cooled down to 25°C and transferred into an ice-water mixture(500 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic extracts were washed with saturated saline solution (50 mL), dried over $MgSO_4$ and concentrated *in vacuo* to yellow oil **2** which could be used in the

next step without further purification.

A solution of **2** (4 g, 23.8 mmol) in DMF (12 mL) was treated with Li_2CO_3 (3.6g, 48.7 mmol) at room temperature. The resulting mixture was then heated to 120 °C for 7 h. Then the reaction mixture was cooled down to 25°C and transferred into an ice-water mixture(500 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic extracts were washed with saturated saline solution (50 mL), dried over $MgSO_4$, concentrated and purified by flash chromatography (Ethyl Acetate/Petroleum Ether = 1:1). The product was obtained as a light brown solid **3** (1.1 g, 35.1%).

m.p. 70-72 °C, ^{1}H-NMR (300 MHz, CDCl$_3$)δ: 7.05 (s, 1 H, NHCO), 6.78(t, J = 4.2 Hz, 1 H, =CH), 3.48 (td, J_1 = 2.7 Hz, J_2 = 7.2 Hz, 2 H, NCH$_2$), 2.44-2.50 (m, 2 H, CH$_2$); MS (ESI): 132.2 [M+H]$^+$.

5.3 3-morpholino-5,6-dihydropyridin-2(1*H*)-one (4)

A solution of **3** (1.3 g, 100.0 mmol) and morpholine in DMF (5 mL) was treated with Li_2CO_3 (1.5g, 20.3 mmol) at room temperature. The resulting mixture was then heated at 130 °C for 3 h. Then the reaction mixture was cooled down to 25°C and filtered. The filtrate was concentrated and the residue was purified by flash chromatography (Ethyl Acetate/Petroleum Ether = 1:2). The product was obtained as a brown solid **4** (0.6g, 30.2%).

M.p. 123-126 °C, ^{1}H-NMR(CDCl$_3$, 300 MHz)δ: 6.15(s, 1 H, NH), 5.56(t, J = 1.5 Hz, 1 H, =CH), 3.82-3.85 (m, 4 H, 2 CH$_2$), 3.30-3.36 (m, 2 H, CH$_2$), 2.87-2.90 (m, 4 H, 2 CH$_2$), 2.33-2.40 (m, 2 H, CH$_2$); MS (ESI): 183.1 [M+H]$^+$.

5.4 3-bromo-5,6-dihydropyridin-2(1*H*)-one(6)

A solution of piperidin-2-one (9.9 g, 100.0 mmol) in CH_2Cl_2 (200 mL) was treated with PCl_5 (41.6 g, 200.0 mmol) at 0-5 °C for 10 min. Then ZnI_2 (1.0 g, 3.0 mmol) was added under N_2 and warmed up to room temperature for 1 h. Then Br_2 (32.0 g, 200.0 mmol) in CH_2Cl_2 (100 mL) was slowly added, the reaction mixture was stirred for 12 h and transferred into an ice-water mixture(500 mL). The aqueous layer was extracted with CH_2Cl_2(5 × 30 mL). The combined organic extracts were washed with saturated saline solution (50 mL), dried over $MgSO_4$, concentrated and purified by flash chromatography (Ethyl Acetate/Petroleum Ether= 1:4). The product was obtained as a light brown solid **5** (21.0g, 82%).

A solution of **5** (12.8 g, 50.0 mmol) in DMF (70 mL) was treated with LiCl (4.0 g, 51.0 mmol) and Li_2CO_3 (7.0 g, 95.0 mmol) at room temperature. The resulting mixture was then heated at 130 °C for 8 h. Then the reaction mixture was concentrated and purified by flash chromatography (Ethyl Acetate/Petroleum Ether = 1:2). The product was obtained as a light brown solid **6** (6.8 g, 77%).

M.p. 80-82 °C, ^{1}H-NMR (CDCl$_3$, 300 MHz) δ: 7.05 (t, J = 1.5 Hz, 1 H, =CH), 6.89 (s, 1 H, NH), 3.49 (t, J = 7.2 Hz, 2 H, NCH$_2$), 2.40-2.46 (m, 2 H, CH$_2$); MS (ESI): 176.3 [M+H]$^+$.

5.5 General Procedure for the synthesis of 9a – 9d:

A solution of 3,4,5-trimethoxybenzaldehyde**7** (2.0 g, 10.0 mmol), piperidine (0.2 g, 2.0 mmol) and 2-substitued malonic acid (5.9 g, 57.0 mmol) in dry pyridine (20 mL) was refluxed for 24 h. Then the reaction mixture was concentrated and water (50 mL) was added slowly. The solution was extracted with ethyl acetate (3 × 20 mL). The

combined organic extracts were washed with saturated saline solution (50 mL), dried over MgSO$_4$, concentrated and purified by flash chromatography (Ethyl Acetate/Petroleum Ether = 1:2) to afford **9a – 9d**.

5.5.1 (*E*)-2-methyl-3-(3,4,5-trimethoxyphenyl)acrylic acid (9a):

White solid, yield 66.1%, m.p. 154-156 °C, ^{1}H-NMR (CDCl$_3$, 300 MHz) δ: 7.77 (s, 1 H, =CH),6.69 (s, 2 H, Ph), 3.90 (s, 9 H, 3 OMe), 2.19 (s, 3 H, Me); MS (ESI): 253.2 [M+H]$^+$.

5.5.2 (*E*)-2-ethyl-3-(3,4,5-trimethoxyphenyl)acrylic acid (9b):

Off-white solid, yield 53.0%, m.p. 120-122 °C, ^{1}H-NMR (CDCl$_3$, 500 MHz) δ: 7.74 (s, 1 H, =CH), 6.69 (s, 2 H, Ph), 3.91 (s, 3 H, OMe), 3.90 (s, 6 H, 2 OMe), 2.64 (q, J = 7.5 Hz, 2 H, CH$_2$), 1.27 (t, J = 7.5 Hz, 3 H, Me); MS (ESI): 267.1 [M+H]$^+$.

5.5.3 (*E*)-2-propyl-3-(3,4,5-trimethoxyphenyl)acrylic acid (9c):

Off-white solid, yield 36.0%, m.p. 97-99 °C, ^{1}H-NMR (CDCl$_3$, 500 MHz) δ: 7.73 (s, 1 H, =CH), 6.67 (s, 2 H, Ph), 3.90 (s, 3 H, OMe), 3.88 (s, 6 H, 2 OMe), 2.50-2.58 (m, 2 H, CH$_2$), 1.64-1.69 (m, 2 H, CH$_2$), 1.03 (t, J = 7.5 Hz, 3 H, Me); MS (ESI): 281.4 [M+H]$^+$.

5.5.4 (*E*)-2-butyl-3-(3,4,5-trimethoxyphenyl)acrylic acid (9d):

Off-white solid, yield 33.2%, m.p. 94-96 °C, ^{1}H-NMR (CDCl$_3$, 300 MHz) δ: 7.72 (s, 1 H, =CH),6.67 (s, 2 H, Ph), 3.89 (s, 3 H, OMe), 3.88 (s, 6 H, 2 OMe), 2.56-2.61 (m, 2 H, CH$_2$, butyl), 1.58-1.66 (m, 2 H, CH$_2$, butyl), 1.40-1.48 (m, 2 H, CH$_2$, butyl), 0.95 (t, J = 7.5 Hz, 3 H, Me); MS (ESI): 295.2 [M+H]$^+$.

5.6 General Procedure for synthesis of 11a – 11p:

A solution of substitued (*E*)-3-(3,4,5-trimethoxyphenyl)acrylic acid (**9a – 9e**) and

oxalyl chloride (3.89 eq.) in dried THF was refluxed for 4 h under N_2. The reaction was cooled to 0°C and triethylamine (2.00 eq.) was slowly added. The reaction solution was stirred for 15 min at 0 ℃. Then **3**, **4** or **6** (1.50 eq.) in dried THF was slowly added and stirred at room temperature for 12 h. The mixture was diluted with CH_2Cl_2 (30 mL) and washed with saturated ammonium chloride solution (20 mL) and saturated saline solution (20 mL), dried over $MgSO_4$, concentrated and purified by flash chromatography (Ethyl Acetate/Petroleum Ether = 1:2) to afford **11a – 11p**.

5.6.1(*E*)-1-(2-methyl-3-(3,4,5-trimethoxyphenyl)acryloyl)-5,6-dihydropyridin-

-2(1*H*)-one (11a):

Light yellow solid, Yield 75.3%, m.p. 91-92 °C, ^{1}H-NMR (CDCl$_3$, 300 MHz) δ: 6.95-6.98 (m, 1 H, =CH), 6.87 (s, 1 H, =CH), 6.64 (s, 2 H, Ph), 6.02 (d, J = 9.6 Hz, 1 H, =CH), 3.94 (t, J = 6.6 Hz, 3 H, NCH$_2$), 3.88 (s, 9 H, 3 OMe), 2.54-2.59 (m, 2 H, CH$_2$); 2.16 (s, 3 H, CH$_3$); ^{13}C-NMR (CDCl$_3$, 300 MHz) δ: 168.92, 165.90, 153.37, 145.46, 143.83, 130.66, 125.85, 121.09, 105.47, 60.98, 56.18, 41.66, 29.70, 24.81; HRMS (ESI) calculated for (C18H21NO5): 331.1447, found 331.1434.

5.6.2(*E*)-1-(2-ethyl-3-(3,4,5-trimethoxyphenyl)acryloyl)-5,6-dihydropyridin-

-2(1*H*)-one (11b):

Off-white solid, yield 71.6%, m.p. 96-98 °C, ^{1}H-NMR (CDCl$_3$, 600 MHz) δ: 6.91-6.94 (m, 1 H, =CH), 6.70 (s, 1 H, =CH), 6.56 (s, 2 H, Ph), 5.99 (dt, J_1 = 9.6 Hz, J_2 = 1.8 Hz, 1 H, =CH), 3.93 (t, J = 6.6 Hz, 2 H, NCH$_2$), 3.85 (s, 9 H, 3 OMe), 2.63 (q, J = 7.8 Hz, 2 H, CH$_2$), 2.51-2.54 (m, 2 H, CH$_2$), 1.13 (t, J = 7.8 Hz, 3 H, Me);

^{13}C-NMR (CDCl$_3$, 300 MHz) δ: 175.72, 165.22, 152.99, 145.34, 141.23, 137.68,

131.84, 131.65, 125.38, 106.21, 60.90, 56.08, 42.66, 24.76, 22.44, 13.03; HRMS (ESI)

calculated for (C19H23NO5): 345.1576, found 345.1574.

5.6.3 (*E*)-morpholino-1-(3-(3,4,5-trimethoxyphenyl)acryloyl)-5,6-dihydro-pyridin-2(1*H*)-one (11c):

Brown oil, yield 45.0%, ^{1}H-NMR (CDCl$_3$, 600 MHz) δ: 7.67 (d, *J* = 15.0 Hz, 1 H, =CH), 7.45 (d, *J* = 15.0 Hz,1 H, =CH), 6.80 (s, 2 H, Ph), 6.18-6.20 (m, 1 H, =CH), 3.94 (t, J = 6.6 Hz , 2 H, NCH$_2$), 3.87-3.89 (m, 13 H, 3 OMe + 2 CH$_2$), 2.94-2.97 (m, 4 H, 2 CH$_2$), 2.45-2.48 (m, 2 H, CH$_2$); ^{13}C-NMR (CDCl$_3$, 300 MHz) δ:169.36, 163.08, 153.31, 143.80, 143.64, 140.03, 130.54, 121.33, 120.76, 105.51, 66.59, 60.88, 56.17, 50.70, 42.16, 23.38 ； HRMS (ESI) calculated for (C21H26N2O6): 402.1791, found 402.1783.

5.6.4 (*E*)-morpholino-1-(2-methyl-3-(3,4,5-trimethoxyphenyl)acryloyl)-5,6-di-hydropyridin-2(1*H*)-one (11d):

Light yellow solid, yield 67.8%, m.p. 127-129 °C, ^{1}H-NMR(CDCl$_3$, 600 MHz) δ: 6.86 (s, 1 H, =CH), 6.63 (s, 2 H, Ph), 5.99-5.60 (m ,1 H, =CH), 3.80-3.90 (m, 15 H, NCH$_2$ + 3 OMe + 2 CH$_2$), 2.88-2.89 (m, 4 H, 2 CH$_2$), 2.50-2.53 (m, 2 H, CH$_2$), 2.16 (s, 3 H, Me); ^{13}C-NMR (CDCl$_3$, 300 MHz) δ:176.53, 162.61, 152.92, 143.38, 137.97, 134.54, 133.45, 131.59, 120.39, 106.98, 66.61, 60.92, 56.15, 50.70, 43.41, 23.49, 15.85; HRMS (ESI) calculated for (C22H28N2O6): 416.1947, found 416.1938.

5.6.5 (*E*)-morpholino-1-(2-ethyl-3-(3,4,5-trimethoxyphenyl)acryloyl)-5,6-dihydro-pyridin-2(1*H*)-one (11e):

Light yellow solid, yield 67.8%, m.p. 149-151°C, ^{1}H-NMR(CDCl$_3$, 600 MHz) δ: 6.70

(s, 1 H, =CH), 6.58 (s, 2 H, Ph), 5.92 (t, J = 4.8 Hz ,1 H, =CH), 3.84-3.88 (m, 11 H,

NCH$_2$ + 3 OMe), 3.81 (t, J = 4.8 Hz , 4 H, 2 CH$_2$), 2.86 (t, J = 4.8 Hz, 4 H, 2 CH$_2$),

2.65 (q, J = 7.2 Hz, 2 H, CH$_2$), 2.50 (q, J = 6.6 Hz , 2 H, CH$_2$), 1.12 (t, J = 7.2 Hz , 3

H, CH$_3$); ^{13}C-NMR (CDCl$_3$, 300 MHz) δ:176.01, 162.63, 152.97, 143.51, 141.44,

137.81, 131.93, 131.58, 120.23, 106.44, 66.64, 60.93, 56.14, 50.69, 43.13, 23.49,

22.47, 13.00; HRMS (ESI) calculated for (C23H30N2O6): 430.2104, found 430.2107.

5.6.6 (*E*)-morpholino-1-(2-propyl-3-(3,4,5-trimethoxyphenyl)acryloyl)-5,6-di-

hydropyridin-2(1*H*)-one (11f):

Yellow solid, yield 59.7%, m.p. 124-126 °C, ^{1}H-NMR (CDCl$_3$, 600 MHz) δ: 6.74 (s, 1

H, =CH), 6.58 (s, 2 H, Ph), 5.90 (t , J = 4.8 Hz ,1 H, =CH), 3.80-3.90 (m, 15 H, NCH$_2$

+ 3 OMe + 2 CH$_2$), 2.85 (t , J = 4.2 Hz ,4 H, 2 CH$_2$), 2.60 (t , J = 4.2 Hz, 2H, CH$_2$),

2.50 (q, J = 6.0 Hz , 2 H, CH$_2$), 1.54-1.58 (m, 2 H, CH$_2$), 0.94 (t, J = 7.2 Hz , 3 H,

CH$_3$); ^{13}C-NMR (CDCl$_3$, 300 MHz) δ: 176.30, 162.58, 152.97, 143.56, 140.23, 137.83,

132.63, 131.60, 120.13, 106.48, 66.63, 60.94, 56.12, 50.69, 43.25, 31.45, 23.49, 22.10,

14.36; HRMS (ESI) calculated for (C24H32N2O6): 444.2260, found 444.2251.

5.6.7 (*E*)-3-Cloro-1-(3-(3,4,5-trimethoxyphenyl)acryloyl)-5,6-dihydro-pyridin-

-2(1*H*)-one (11g):

Yellow solid, yield 56.0%, m.p. 140-144 °C, ^{1}H-NMR (CDCl$_3$, 600 MHz) δ: 7.73 (d,

J = 15.6 Hz, 1 H, =CH),7.45 (d, J = 15.6 Hz, 1 H, =CH), 7.11 (t, J = 4.8 Hz, 1 H,

=CH), 6.83 (s, 2 H, Ph), 4.12 (t, J = 6.6 Hz, 2 H, NCH$_2$), 3.90-3.92 (m, 9 H, 3 OMe),

2.57-2.60 (m, 2 H, CH$_2$); ^{13}C-NMR (CDCl$_3$, 300 MHz) δ: 168.52, 161.42, 153.40,

145.02, 141.18, 140.24, 130.37, 128.21, 120.23, 105.64, 60.98, 56.23, 41.80, 25.30;

HRMS (ESI) calculated for (C17H18ClNO5): 351.0874, found 351.0863.

5.6.8 (*E*)-3-Cloro-1-(2-methyl-3-(3,4,5-trimethoxyphenyl)acryloyl)-5,6-dihydro-pyridin-2(1*H*)-one (11h):

Light yellow solid, yield 53.3%, m.p. 105-108 °C, ^{1}H-NMR (CDCl$_3$, 300 MHz) δ: 7.10 (t, *J* = 4.5 Hz, 1 H, =CH), 6.90 (s, 1H, =CH), 6.63 (s, 2 H, Ph), 3.96 (t, *J* = 6.3 Hz, 2 H, NCH$_2$), 3.87 (s, 9 H, 3 OMe), 2.64 (q, *J* = 6.3 Hz, 2 H, CH$_2$), 2.15 (s, 3 H, CH$_3$); ^{13}C-NMR (CDCl$_3$, 300 MHz) δ: 175.71, 160.80, 152.98, 140.88, 138.06, 134.26, 133.87, 131.37, 127.84, 106.88, 60.93, 56.17, 43.16, 25.33, 15.62; HRMS (ESI) calculated for (C18H20ClNO5): 365.1030, found 365.1015.

5.6.9 (*E*)-3-Cloro-1-(2-ethyl-3-(3,4,5-trimethoxyphenyl)acryloyl)-5,6-dihydro-pyridin-2(1*H*)-one (11i):

Off white solid, yield 49.7%, m.p. 127-129 °C, ^{1}H-NMR (CDCl$_3$, 600 MHz) δ: 7.07 (t, *J* = 4.8 Hz, 1 H, =CH), 6.74 (s, 1 H, =CH), 6.56 (s, 2 H, Ph), 3.96 (t, *J* = 6.6 Hz, 2 H, NCH$_2$), 3.86 (s, 3 H, OMe), 3.85 (s, 6 H, 2 OMe), 2.61-2.68 (m, 4 H, 2 CH$_2$), 1.14 (t, *J* = 7.2 Hz, 3 H, CH$_3$); ^{13}C-NMR (CDCl$_3$, 300 MHz) δ: 175.42, 160.92, 153.03, 140.78, 140.60, 137.92, 132.93, 131.33, 127.96, 106.31, 60.93, 56.15, 43.00, 25.34, 22.37, 13.05; HRMS (ESI) calculated for (C19H22ClNO5): 379.1187, found 379.1187.

5.6.10 (*E*)-3-Cloro-1-(2-propyl-3-(3,4,5-trimethoxyphenyl)acryloyl)-5,6-dihydro-pyridin-2(1*H*)-one (11j):

Off white solid, yield 41.6%, m.p. 121-123 °C, ^{1}H-NMR (CDCl$_3$, 300 MHz) δ: 7.08 (t, *J* = 4.8 Hz, 1 H, =CH), 6.78 (s, 1 H, =CH), 6.57 (s, 2 H, Ph) , 3.96 (t, *J* = 6.3 Hz, 2 H,

NCH$_2$), 3.86 (s, 3 H, OMe), 3.85 (s, 6 H, 2 OMe), 2.56-2.66 (m, 4 H, 2 CH$_2$),

1.56-1.61 (m, 2 H, CH$_2$), 0.96 (t, J = 7.2 Hz, 3 H, CH$_3$); ^{13}C-NMR (CDCl$_3$, 300 MHz)

δ: 175.59, 160.88, 153.00, 140.81, 139.31, 137.95, 133.53, 131.33, 127.88, 106.36,

60.88, 56.09, 43.06, 31.24 , 25.30, 22.09, 14.31; HRMS (ESI) calculated for

(C20H24ClNO5): 393.1343, found 393.1354.

5.6.11 (*E*)-3-Cloro-1-(2-butyl-3-(3,4,5-trimethoxyphenyl)acryloyl)-5,6-dihydro-

pyridin-2(1*H*)-one (11k):

Off white solid, yield 47.3%, m.p. 83-85°C, ^{1}H-NMR (CDCl$_3$, 300 MHz) δ: 7.08 (t, J

= 4.5 Hz, 1 H, =CH), 6.77 (s, 1 H, =CH), 6.57 (s, 2 H, Ph), 3.97 (t, J = 6.3 Hz,2 H,

NCH$_2$), 3.86 (s, 9 H, 3 OMe), 2.58-2.66 (m, 4 H, 2 CH$_2$), 1.53-1.62 (m, 2 H, CH$_2$),

1.32-1.40 (m, 2 H, CH$_2$), 0.89 (t, J =7.2Hz, 3H, Me); ^{13}C-NMR (CDCl$_3$, 300 MHz) δ:

175.67, 160.91, 153.03, 140.72, 139.46, 137.94, 133.43, 131.36, 127.99, 106.33,

60.93, 56.12, 43.08, 30.91, 29.02, 25.35, 23.01, 13.95; HRMS (ESI) calculated for

(C21H26ClNO5): 407.1500, found 407.1490.

5.6.12 (*E*)-3-Bromo-1-(3-(3,4,5-trimethoxyphenyl)acryloyl)-5,6-dihydropyridin-

-2(1*H*)-one (11l):

Yellow solid, yield 73.2%, m.p. 138-144 °C, ^{1}H-NMR (CDCl$_3$, 300 MHz) δ: 7.71 (d,

1 H, J = 15.3 Hz, =CH), 7.41 (d, 1 H, J = 15.3 Hz, =CH),7.36 (t, J=4.5Hz, 1 H, =CH),

6.81 (s, 2 H, Ph) , 4.11 (t, J = 6.3 Hz , 2 H, NCH$_2$), 3.90 (s, 6 H, 2 OMe), 3.89 (s, 3 H,

OMe), 2.50-2.56 (m, 2 H, CH$_2$); ^{13}C-NMR (CDCl$_3$, 300 MHz) δ: 168.63, 161.12,

153.41, 145.95, 145.04, 140.26, 130.39, 120.25, 119.14, 105.68, 60.99, 56.26, 41.91,

26.72 ； HRMS (ESI) calculated for (C17H18BrNO5): 395.0368, found 395.0371.

5.6.13 (*E*)-3-Bromo-1-(2-methyl-3-(3,4,5-trimethoxyphenyl)acryloyl)-5,6-di-

hydropyridin-2(1*H*)-one (11m):

Light yellow solid, yield 65.3%, m.p. 99-102 °C, ^{1}H NMR (CDCl$_3$, 300 MHz) δ: 7.36

(t, *J* = 4.5 Hz, 1 H, =CH), 6.89 (s, 1H, =CH), 6.63 (s, 2 H, Ph), 3.97 (t, *J* = 6.3 Hz, 2

H, NCH$_2$), 3.87 (s, 9 H, 3 OMe), 2.57-2.63 (m, 2 H, CH$_2$), 2.15 (s, 3 H, Me);

^{13}C-NMR (CDCl$_3$, 300 MHz) δ: 175.84, 160.54, 152.98, 145.64, 138.09, 134.23,

131.38, 133.89, 118.68, 106.93, 60.93, 56.19, 43.24, 26.73, 15.65; HRMS (ESI)

calculated for (C18H20BrNO5): 409.0525, found 409.0510.

5.6.14 (*E*)-3-Bromo-1-(2-ethyl-3-(3,4,5-trimethoxyphenyl)acryloyl)-5,6-dihydro-

pyridin-2(1*H*)-one (11n):

Yellow solid, yield 67.1%, m.p 85-87 °C, ^{1}H NMR: (CDCl$_3$, 300 MHz) δ: 7.36 (t, *J* =

4.5 Hz, 1 H, =CH), 6.76 (s, 1 H, =CH), 6.58 (s, 2 H, Ph), 3.99 (t, *J* = 6.3 Hz,2 H,

NCH$_2$), 3.88 (s, 9 H, 3 OMe), 2.57-2.69 (m, 4 H, 2 CH$_2$), 1.16 (t, *J* = 7.5 Hz, 3 H, Me);

^{13}C-NMR (CDCl$_3$, 300 MHz) δ: 175.53, 160.65, 153.02, 145.57, 140.58, 137.92,

132.95, 131.33, 118.80, 106.33, 60.93, 56.14, 43.08, 26.73, 22.36, 13.05; HRMS (ESI)

calculated for (C19H22BrNO5): 423.0681, found 423.0688.

5.6.15 (*E*)-3-Bromo-1-(2-propyl-3-(3,4,5-trimethoxyphenyl)acryloyl)-5,6-dihydro-

pyridin-2(1*H*)-one (11o):

Off white solid, yield 56.4%, m.p 80-82°C, ^{1}H NMR: (CDCl$_3$, 300 MHz) δ: 7.35 (t, *J*

= 4.5 Hz, 1 H, =CH), 6.77 (d, *J* = 3.3 Hz, 1 H, =CH), 6.57 (s, 2 H, Ph), 3.97 (t, *J* = 6.3

Hz, 2 H, NCH$_2$), 3.87 (s, 3 H, OMe), 3.86(s, 6 H, 2 OMe), 2.56-2.62 (m, 4 H, 2 CH$_2$),

1.56-1.61 (m, 2 H, CH$_2$), 0.87-0.97 (m, 3 H, Me); ^{13}C-NMR (CDCl$_3$, 300 MHz) δ:

175.75, 160.63, 153.00, 145.56, 139.36, 137.91, 133.57, 133.42, 118.79, 106.33,

60.93, 56.11, 43.16, 29.02, 26.72, 23.01, 13.93;HRMS (ESI) calculated for

(C20H24BrNO5): 437.0838, found 437.0821.

5.6.16 (*E*)-3-Bromo-1-(2-butyl-3-(3,4,5-trimethoxyphenyl)acryloyl)-5,6-dihydro-

pyridin-2(1*H*)-one (11p):

Off white solid, yield 49.7%, m.p.109-112 °C, ^{1}H NMR: (CDCl$_3$, 300 MHz) δ: 7.35 (t,

J = 4.5 Hz, 1 H, =CH), 6.77 (s, 1 H, =CH), 6.58 (s, 2 H, Ph), 3.98 (t, J = 6.3 Hz, 2 H,

NCH$_2$), 3.89 (s, 9 H, 3 OMe), 2.56-2.63 (m, 4 H, 2 CH$_2$), 1.54-1.59 (m, 2 H, CH$_2$),

1.29-1.40 (m, 2 H, CH$_2$), 0.90 (t, J = 7.2 Hz, 3 H, Me); ^{13}C-NMR (CDCl$_3$, 300 MHz)

δ: 175.78, 160.66, 153.00, 145.55, 139.44, 138.31, 133.44, 131.36, 118.80, 106.33,

60.94, 56.11, 43.17, 30.90, 29.03, 26.74, 23.02, 13.94; HRMS (ESI) calculated for

(C21H26BrNO5): 451.0994, found 451.0980.

In Vitro **Cytotoxicity Assay.** Cells were plated in 96-well microtiter plates at a

density of 5 × 10^3/well and incubated in a humidified atmosphere with 5% CO$_2$ at

37 °C for 24 h. Test compounds were added to triplicate wells with different

concentrations and 0.1% DMSO for control. After incubation for 72 h, 20 μL of MTT

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/mL)

was added to each well and the plate was incubated for an additional 4 h. The

formazan was dissolved in 100 μL of DMSO. The absorbance (OD) was read on a

WellscanMK-2 microplate reader (Labsystems) at 570 nm. The concentration causing

50% inhibition of cell growth (IC$_{50}$) was determined by the Logit method.

ROS assay. Cells were treated with compound **11h** and DMSO for 1 hour and ROS

generation was detected with 2'-,7'-dichlorofluorescein diacetate (DCFH-DA) (Invitrogen, Carlsbad, CA, USA). Cells were incubated with 10 μM of DCFH-DA for 30 min at 37°C, washed twice with PBS and immediately analyzed by a fluorescence microscope.

***In vivo* antitumor activity.** The compound was dissolved in normal saline using tween-80 as solubilizer. BALB/C nude male mice (Certificate SCXK-2007-0005, weighing 18 g to 20 g) were obtained from Shanghai Experimental Animal Center, Chinese Academy of Sciences. A549 lung cancer cell suspensions were implanted subcutaneously into the right axilla region of mice. Treatment was begun when implanted tumors had reached a volume of about 100-300 mm^3 (after 17 days). The animals were randomized into appropriate groups (six animals/treatment and eight animals for the control group) and administered by ip injection for fourteen consecutive days after implantation of cells. Tumor volumes were monitored by caliper measurement of the length and width and calculated using the formula of TV = $1/2 \times a \times b^2$, where a is the tumor length and b is the width. Tumor volumes and body weights were monitored every 4 days over the course of treatment. Mice were sacrificed on day 30 to 33 after implantation of cells and tumors were removed and recorded for analysis.

ACKNOWLEDGEMENTS

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Figure Captions

Figure 1. Structure of piperlongumine (PL)

Figure 2. Piperlongumine derivative **11h** (10 μM) induces ROS elevation in A549 cells for 1h. A, control; B, compound **11h**; C, mean fluorescence of control and **11h**

Figure 3. Growth of sc implanted human xenograft in nude mice treated with vehicle, **PL** (ip×14, 2 mg/Kg), **11h** (ip×14, 2 mg/Kg) and DOX (iv×1, 10 mg/Kg) in A549 lung cancer xenograft model. Mean ± SE for group of 6 mice ($P<0.05$).

Table 1. *In vitro* antitumor activity of 2 and 7-substituted piperlongumine derivatives

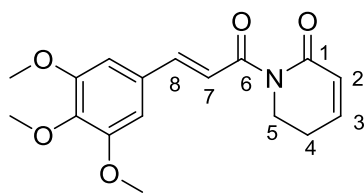**Table 2**. *In vitro* antitumor activity of 2-halogen substituted piperlongumine derivatives

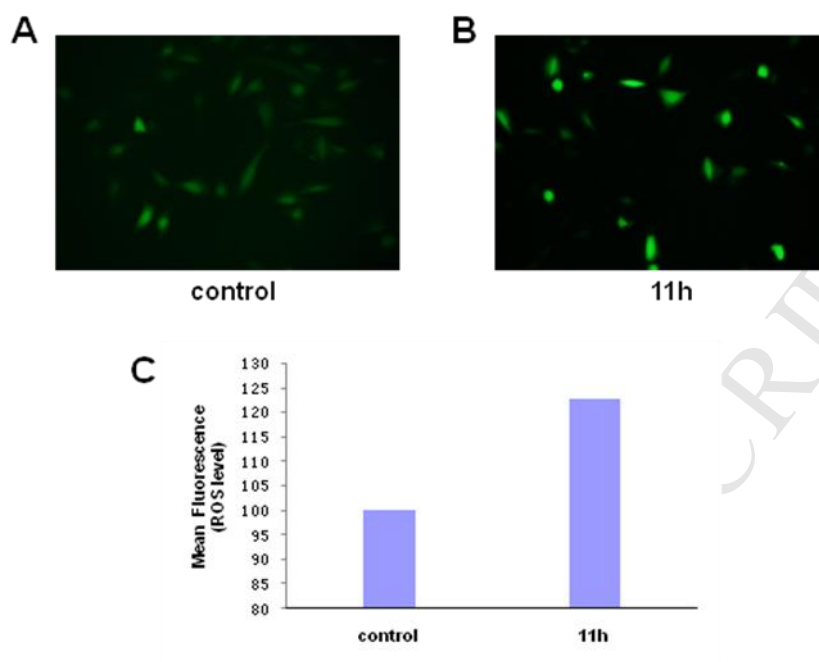**Fig. 1.** Structure of piperlongumine (PL)

Fig. 2. Piperlongumine derivative **11h** (10 μM) induces ROS elevation in A549 cells for 1h. A,

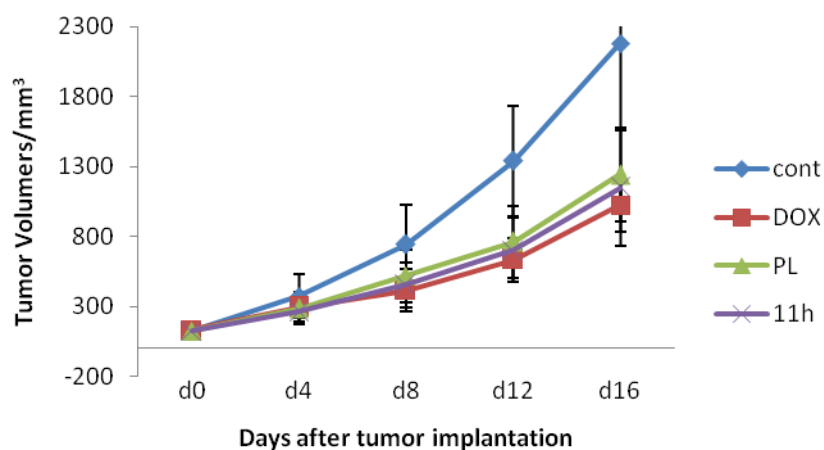control; B, compound **11h**; C, mean fluorescence of control and **11h**

Fig. 3. Growth of sc implanted human xenograft in nude mice treated with vehicle, **PL** (ip×14, 2 mg/Kg), **11h** (ip×14, 2 mg/Kg) and DOX (iv×1, 10 mg/Kg) in A549 lung cancer xenograft model. Mean ± SE for group of 6 mice ($P<0.05$).

Table 1. *In vitro* antitumor activity of 2 and 7-substituted piperlongumine derivatives

Compds	IC$_{50}$ ^{a} (μM)				
	A549	HCT116	ZR-75-30	MDA-MB-231	MRC-5
PL	22.85	6.04	5.86	8.46	35.04
11a	23.95	10.34	3.32	11.71	19.37
11b	17.95	12.34	3.16	23.67	13.53
11c	>100	47.20	52.64	>100	56.05
11d	>100	>100	>100	>100	>100
11e	>100	>100	>100	>100	>100
11f	>100	>100	>100	>100	52.53
11g	19.83	4.28	4.49	6.71	14.30

^{a} Values were measured with MTT method.

Table 2. *In vitro* antitumor activity of 2-halogen substituted piperlongumine derivatives

Compds	IC$_{50}$ [a] (μM)				
	A549	HCT116	MDA-MB-231	Hep3B	WI38
PL	5.90	21.80	19.53	69.46	26.78
11h	3.94	9.85	6.07	16.69	19.60
11i	4.06	9.17	7.27	19.03	5.48
11j	3.81	8.08	8.13	22.36	10.42
11k	3.78	7.53	6.55	14.75	7.93
11l	4.79	7.12	2.73	8.09	20.65
11m	2.67	6.20	2.47	3.10	7.16
11n	2.18	3.02	2.12	8.12	7.94
11o	2.63	2.14	2.36	5.54	5.02
11p	2.56	2.59	2.44	14.85	9.21

[a] Values were measured with MTT method.
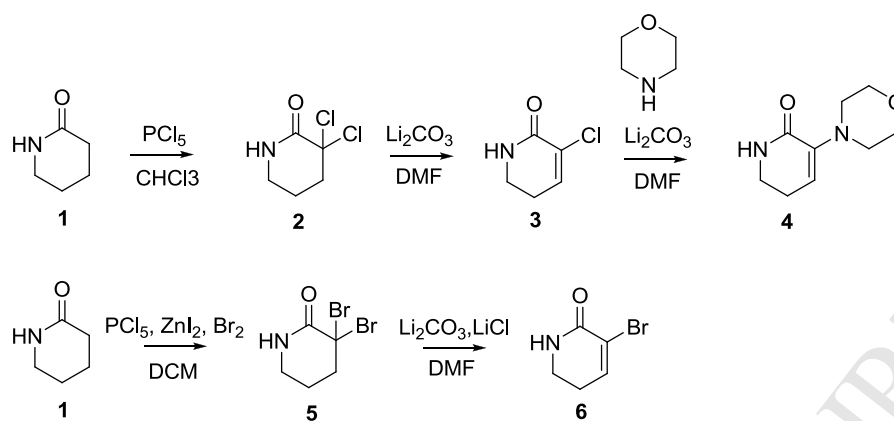
Scheme 1. Synthesis of three key dihydropyridin-2(1*H*)-ones
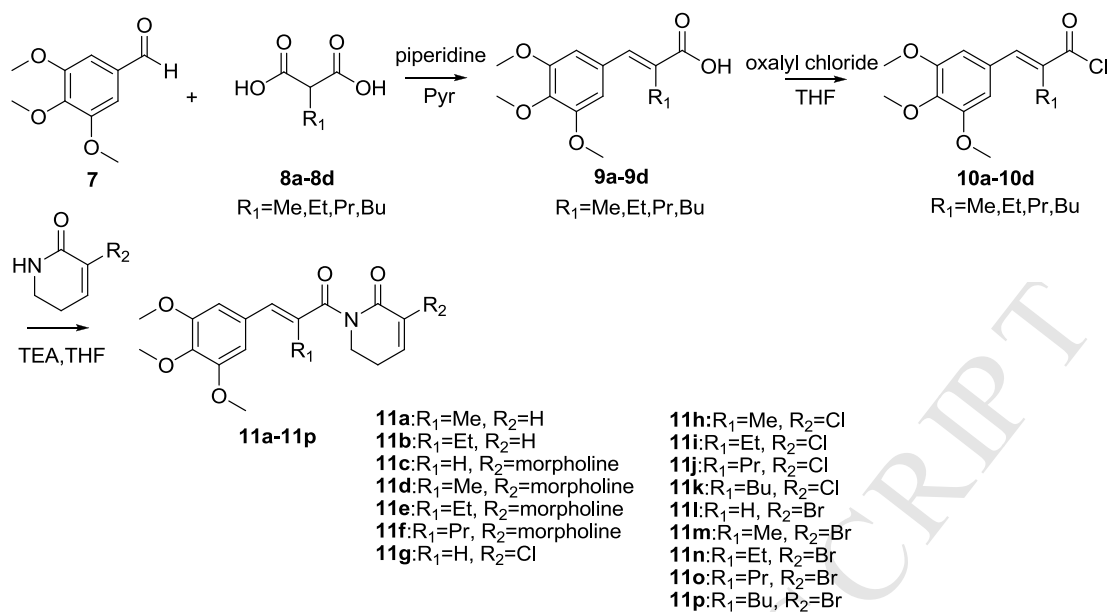
Scheme 2. Synthesis routes of piperlongumine derivatives **11a**-**11p**