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PII: S0223-5234(17)30184-8

DOI: 10.1016/j.ejmech.2017.03.027

Reference: EJMECH 9287

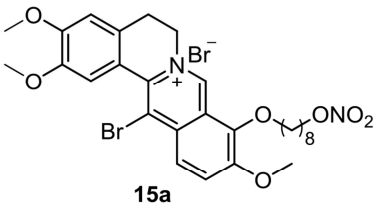
To appear in: *European Journal of Medicinal Chemistry*

Received Date: 24 January 2017

Revised Date: 14 March 2017

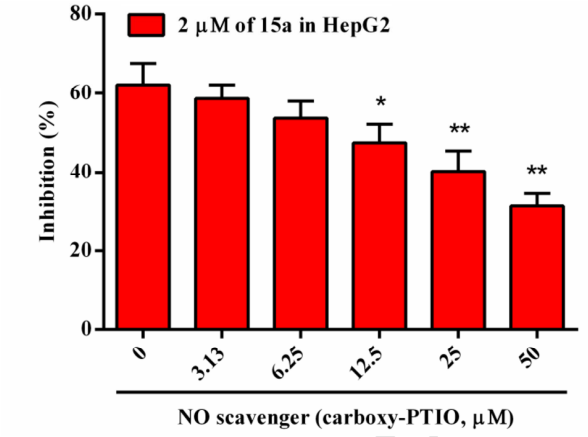
Accepted Date: 15 March 2017

Please cite this article as: J. Chen, T. Wang, S. Xu, A. Lin, H. Yao, W. Xie, Z. Zhu, J. Xu, Design, synthesis and biological evaluation of novel nitric oxide-donating protoberberine derivatives as antitumor agents, *European Journal of Medicinal Chemistry* (2017), doi: 10.1016/j.ejmech.2017.03.027.



15a

IC_{50}: 1.36 μM (HepG2); 1.21 μM (SMMC-7721);
2.29 μM (HCT-116); 1.26 μM (HL-60);
28.93 μM (normal liver LO-2 cells)
In vivo tumor inhib.: 45.9% (15 mg/kg)
(H22 liver cancer) 62.5% (30 mg/kg)



Design, synthesis and biological evaluation of novel nitric oxide-donating protoberberine derivatives as antitumor agents

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Abstract: A novel class of NO-donating protoberberine derivatives were synthesized and initially evaluated for their anti-hepatocellular carcinoma activities. Most of the compounds exhibited more potent activity against HepG2 cells than parent compounds berberine and palmatine. In particular, compound **15a** exerted the strongest activity with an IC_{50} value of 1.36 μM. Moreover, most compounds released moderate levels of NO *in vitro*, and the antitumor activity of **15a** in HepG2 cells was remarkably diminished by an NO scavenger. Interestingly, compound **15a** displayed a broad-spectrum antitumor efficacy and possessed good selectivity between tumor cells (HepG2, SMMC-7721, HCT-116, HL-60) and normal liver LO-2 cells. The mechanism studies revealed that **15a** blocked the G2 phase of the cell cycle and induced apoptosis of HepG2 cells by mitochondrial depolarization. Furthermore, **15a** inhibited tumor growth in H22 liver cancer xenograft mouse model by 62.5% (*w/w*), which was significantly superior to parent compound palmatine (41.6%, *w/w*). Overall, the current study may provide a new approach for the discovery of novel antitumor agents.

Key words: berberine; palmatine; nitric oxide (NO) donor; hybrid compound; antitumor activity

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies around the world, accounting for over 80% of all liver cancers with extremely poor prognosis [1]. The incidence of HCC has exceeded 600,000 cases annually [1], and HCC has become the third leading cause of cancer death in China [2]. Although the current management strategies, including surgical resection, local ablation or liver transplantation, provide effective treatment for a small number of cases in the early stages of HCC [3] while no clinically curable therapy was available for the advanced HCC [4], and a large percentage of advanced HCC do not respond to any conventional chemotherapies, mainly due to the high level of intrinsic and acquired chemo-resistances [5]. Thus, the development of novel and effective therapies for this devastating disease is urgently required.

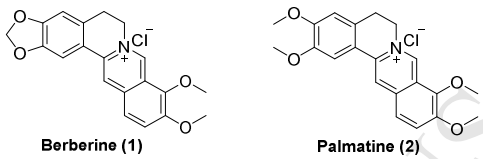


Figure 1. Chemical structures of berberine and palmatine

Many plant-derived agents with excellent characters have been accepted as potential alternatives for the therapy of invasive hepatoma [6]. Alkaloids are well-known to be a group of diverse plant secondary metabolites. Especially the quaternary ammonium salt from the protoberberine group of isoquinoline alkaloids, such as berberine (**1**) and palmatine (**2**) (Figure 1), have been reported to possess multiple pharmacological activities, including antimicrobial, anti-inflammatory, DNA-binding effects, *etc* [7-8]. In recent years, the anticancer activity of berberine in particular has received much more attention and a great deal of efforts have been devoted to the prevention of HCC [9-10]. Studies have shown that berberine inhibited cell proliferation and migration, induced cell apoptosis and autophagy, and caused cell cycle arrest in several HCC cell lines [11-14]. Besides, berberine selectively inhibited hepatoma cell invasion while elicits less cytotoxic effects in normal hepatocytes [15-16]. As a result, berberine seems to be a promising candidate for the treatment of HCC, however, its poor absorption and moderate activity may hamper its further development [17], which stimulates our efforts toward the design and synthesis of berberine and its analogue palmatine derivatives with improved biochemical properties.

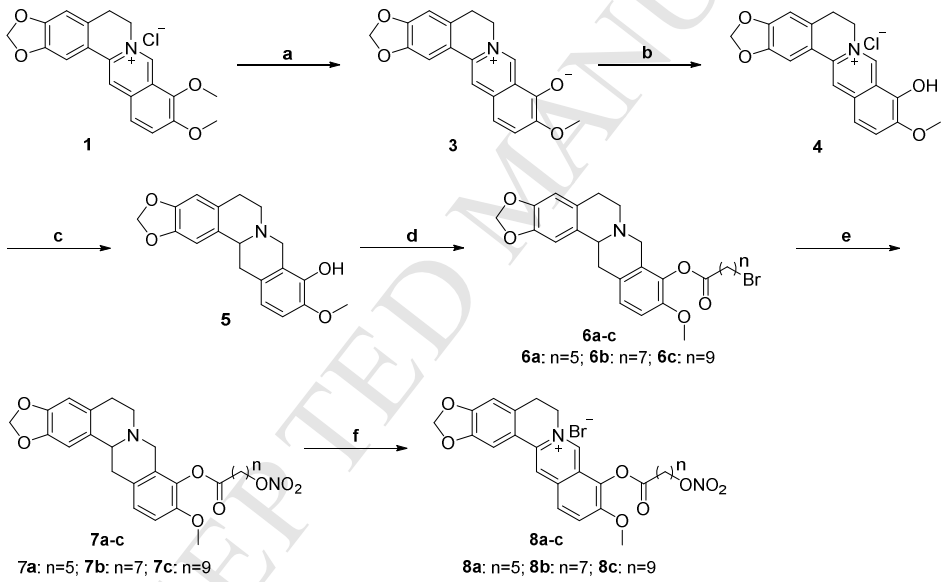
Nitric oxide (NO) is an important signaling and/or effector molecule involved in various physiological and pathophysiological processes [18]. Recent studies suggested that the decreased level of NO in liver tissues might contribute to the development and progression of HCC [19]. Besides, NO donors such as sodium nitroprusside (SNP), effectively suppressed proliferation, migration and invasion, arrested the cell cycle and induced apoptosis in HepG2 cells [20]. It is well known that gaseous NO is generally ineffective when delivered to tumor directly due to its short half-life and chemical instability [21]. Thus, NO donors are usually selected as surrogates for NO in anticancer studies [22]. Numerous reports have demonstrated that NO-donating hybrids possessed greater antitumor activity than sole NO donors, parent drugs and/or their combinations [23-27]. More importantly, NO-donating antitumor drugs usually do not induce any drug resistance in tumor cells [28].

Inspired by these findings, it may be meaningful to design and synthesize a series of berberine and palmatine derivatives hybridizing NO donors for the treatment of HCC. Moreover, considering the hydrophilic nature of quaternary ammonium salts as the leading cause of poor intestine absorption [29], diverse lengths of alkyl linkers are employed to improve their lipophilicity at a decent level. Herein, we report the synthesis, *in vitro* and *in vivo* anti-HCC activity, NO release, and anticancer mechanism for a new class of NO-donating berberine and palmatine derivatives.

2. Results and discussion

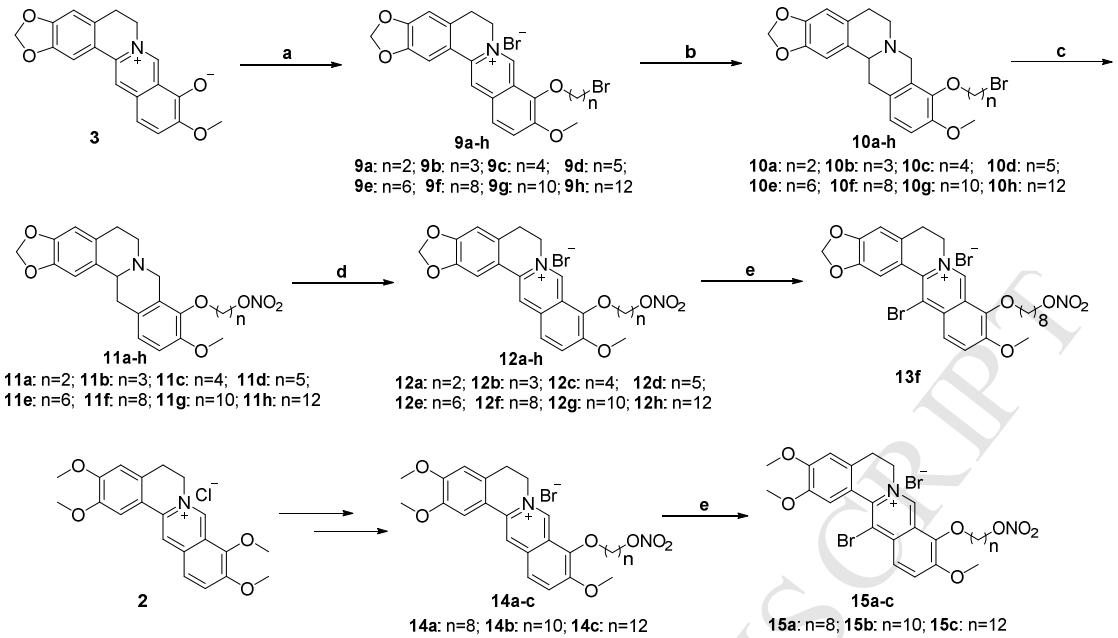
2.1. Chemistry

The synthesis of compounds **8a-c** was illustrated in **Scheme 1**. Selective demethylation of berberine chloride (**1**) through pyrolysis produced compound **3**, followed by acidification to give berberrubine (**4**), which was reduced using $NaBH_4$ to yield compound **5**. Then, esterification of **5** with $Br(CH_2)_nCOOH$ in the presence of EDCI and DMAP gave compounds **6a-c**, which were treated with $AgNO_3$ in dry CH_3CN to obtain compounds **7a-c**, followed by oxidation using NBS (1.5 eq) to afford the title compounds.



Scheme 1. Synthetic routes of the title compounds **8a-c**. Reagents and conditions: (a) 190 ^oC, 20-30 mmHg, 0.5-1 h; (b) HCl, EtOH, rt, 1 h; (c) $NaBH_4$, CH_3OH, rt, 1 h; (d) $Br(CH_2)_nCOOH$, EDCI, DMAP, CH_2Cl_2, rt, 4-8 h; (e) $AgNO_3$, dry CH_3CN, 85 ^oC, 2-4 h; (f) NBS (1.5 eq), $CHCl_3$, 60 ^oC, 1-2 h.

The preparation of compounds **12a-h**, **13f**, **14a-c** and **15a-c** were shown in Scheme 2. Reaction of compound **3** with $Br(CH_2)_nBr$ gave compounds **9a-9h**, which underwent reduction using $NaBH_4$ to form compounds **10a-h,** followed by treatment with $AgNO_3$ in dry CH_3CN to afford compounds **11a-h**. Then, **11a-h** were oxidized using NBS (1.5 eq) to produce compounds **12a-h**, and **12f** was further brominated with NBS (3.0 eq) to provide compound **13f**. Compounds **14a-c** and **15a-c** were obtained from palmatine by the same way.



Scheme 2. Synthetic routes of the title compounds **12a-h, 13f, 14a-c** and **15a-c**. Reagents and conditions: (a) $Br(CH_2)_nBr$, CH_3CN, 80 ^oC, 4-8 h, (b) $NaBH_4$, CH_3OH, rt, 1 h; (c) $AgNO_3$, CH_3CN, 85 ^oC, 2-4 h; (d) NBS (1.5 eq), $CHCl_3$, 60 ^oC, 1 h; (e) NBS (3.0 eq), $CHCl_3$, 60 ^oC, 2-4 h.

2.2. *In vitro* antiproliferative activity in HepG2 cells

Initially, compounds **8a-c** and **12a-h** were prepared and evaluated for their cytotoxicity against HepG2 cells. As shown in Table 1, compounds **8a-c** showed lower activities than the parent compound berberine, suggesting the ester bond linking berberine with NO donors may have a detrimental effect on the activity. When the ester bond was changed to the ether bond, the activity markedly increased (*e.g.* **8a** *vs.* **12e**, **8b** *vs.* **12f**). It was found that the antiproliferative activity gradually strengthened by extending the alkyl chain length from C_2 to C_8 (*e.g.* **12a-f**), however, the chain length had no significant effect on the activity since the C_8 (*e.g.* **12g-h** from C_{10} to C_{12}). Reduction of unsaturated quaternary ammonium to tertiary amine resulted in sharply decreased activity (*e.g.* **12f** *vs.* **11f**), indicating the quaternary ammonium may be essential for the antitumor activity.

Based on the above results, hybrids **14a-c** of palmatine and NO donors with the chain length from C_8 to C_{12} were further synthesized. As expected, the three compounds exhibited remarkably enhanced cytotoxicity compared to palmatine (Table 1). Previous studies indicated that introduction of alkyl chain into C13-position of berberine or palmatine could notably improve the antitumor activity [30] while the corresponding halogenation has rarely been reported. Thus, compounds **13f** and **15a-c** from C13-bromination of **12f** and **14a-c** were further investigated for the structure-activity relationships. It was observed that the bromo-substituted compounds **15a-c** containing palmatine skeleton exhibited slightly increased cytotoxicity against HepG2 cells, while the bromo-substituted compound **13f** containing berberine skeleton showed slightly decreased activity (Table 1). Meanwhile, it was also surprising that introduction of an NO donor into the palmatine skeleton resulted in more improvement on its antiproliferative activity than that in the berberine skeleton, although palmatine possessed much lower actitivity than berberine.

Among these synthesized compounds, compound **15a** bearing palmatine skeleton showed the strongest antiproliferative activity with an IC_{50} value of 1.36 μM, which was superior to that of

positive control cisplatin (IC_{50}, 7.10 μM). As a result, compound **15a** was selected for subsequent anti-HCC mechanism and *in vivo* liver cancer xenograft mouse model study.

Table 1. Antiproliferative activities of the target compounds against HepG2 cells

Compd	IC_{50}[a] (μM)	Compd	IC_{50}[a] (μM)
Berberine	49.01 ± 5.45	**12f**	2.47 ± 0.34
Palmatine	>100	**12g**	4.34 ± 0.27
8a	>100	**12h**	2.52 ± 0.39
8b	>100	**13f**	4.14 ± 0.53
8c	>100	**14a**	3.91 ± 0.46
11f	>100	**14b**	5.59 ± 0.61
12a	25.21 ± 3.78	**14c**	2.55 ± 0.22
12b	22.02 ± 2.64	**15a**	1.36 ± 0.14
12c	18.36 ± 1.41	**15b**	3.01 ± 0.31
12d	12.44 ± 1.05	**15c**	1.98 ± 0.15
12e	8.51 ± 0.93	Cisplatin	7.10 ± 0.48

[a] IC_{50}: concentration of the test compound that inhibits 50% of cell growth. Results are expressed as the mean ± SD (n = 3).

2.3. Effects of NO on antiproliferative activity

To investigate the contribution of NO to the antiproliferative activity, representative compounds (**8b**, **12c**, **12f**, **12h**, **13f**, **14a**, **15a**) were selected to test their NO-releasing abilities. The levels of NO produced by test compounds in the acellular lysates were measured and presented as that of nitrite by Griess assay. As shown in Figure 2A, all of these compounds released moderate amount of NO with time. It was found that the concentrations of released NO slightly increased with the extension of the chain length (*e.g.* **12c** < **12f** < **12h**). Bromination at C13-position of berberine or palmatine had no significant effect on the NO release level (*e.g.* **12f** *vs.***13f**, **14a** *vs.* **15a**). Among them, compounds **8c** and **12c** showed the lowest NO-releasing capacity, which might, at least in part, result in their poor anti-HCC activity. Moreover, the NO-releasing ability of compound **15a** was also verified in the HepG2 cell lysates (Figure 2B).

Furthermore, compound **15a** was examined for the effect of NO on its inhibitory activity against HepG2 cells. After incubation with various concentrations of carboxy-PTIO (PTIO, an NO scavenger) for 2 h, HepG2 cells were treated with 2 μM of **15a** for 72 h. The effects of different treatments on the growth of HepG2 cells were determined by the MTT assay. As seen in Figure 3, treatment with **15a** alone markedly inhibited the growth of HepG2 cells, and this inhibitory effect was decreased by pretreatment with PTIO in a dose-dependent manner. These results suggest that NO generated by **15a** may contribute to its inhibition on HepG2 cells proliferation *in vitro*.

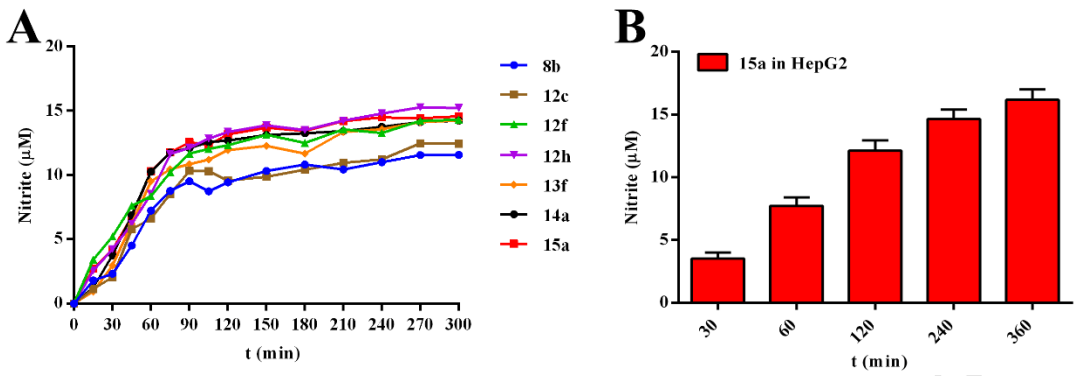


Figure 2. The levels of NO generated by selected compounds: (A) in acellular lysates; (B) in HepG2 cell lysates. The concentrations of released NO for test compounds were measured at 100 μM over time by Griess assay. The individual values were determined by measuring the absorbance at 540 nm and calculated according to the standard curve. Data are expressed as the mean ± SD (n = 3).

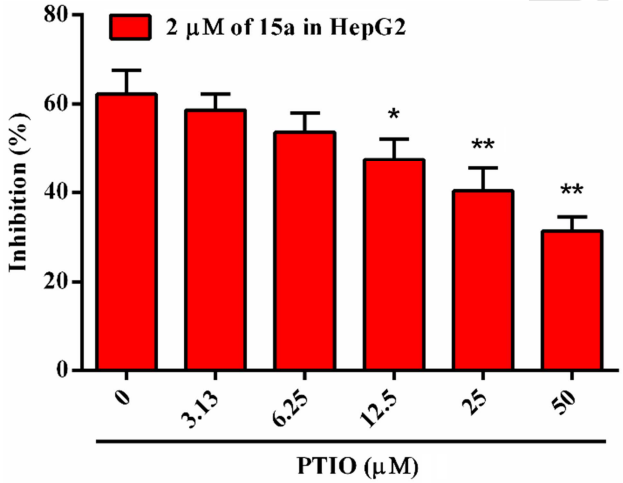


Figure 3. Effect of carboxy-PTIO (PTIO, an NO scavenger) on the antiproliferative activity of **15a**. HepG2 cells were pretreated with the indicated concentrations of PTIO for 2 h and then treated with 2 μM of **15a** for 72 h. Results are expressed as the mean ± SD (n=3). **P* < 0.05, ***P* < 0.01 *vs*. the group without PTIO.

2.4. *In vitro* cytotoxic activities of 12f and 15a against other cells

Compounds **12f** and **15a** were selected to further test their cytotoxic activities against three human cancer cell lines (SMMC-7721: human hepatocellular carcinoma cells; HCT-116: human colorectal carcinoma cells; IL-60: Human promyelocytic leukemia cells) and normal hepatocytes LO-2. As shown in Table 2, the two compounds exhibited markedly enhanced antiproliferative activities against SMMC-7721, HCT-116 and HL-60 cell lines with IC_{50} values ranging from 1.21 to 2.82 μM compared to the parent compounds berberine with IC_{50} values ranging 25.94 to 39.95 μM and palmatine with IC_{50} values of >100 μM, which were even superior to positive control cisplatin with IC_{50} values ranging from 3.71 to 6.50 μM. Interestingly enough, compounds **12f** and **15a** displayed lower cytotoxicity against normal LO-2 cells with IC_{50} values of 33.78 and 28.93 μM, respectively. The results indicated that these NO-donating berberine and palmatine derivatives had a broad-spectrum antitumor activity and possessed good selectivity

between tumor cells and normal liver cells, and would deserve further mechanism and *in vivo* studies.

Table 2. The cytotoxic activities of **12f** and **15a** against four cell lines

compd	IC_{50}[a] (μM)			
	SMMC-7721	HCT-116	HL-60	LO-2
Berberine	25.94 ± 4.63	39.95± 5.72	27.42 ± 4.17	71.55 ± 6.83
Palmatine	>100	>100	>100	>100
12f	2.82 ± 0.57	1.58 ± 0.45	1.42 ± 0.12	33.78 ± 4.74
15a	1.21 ± 0.13	2.29 ± 0.32	1.26 ± 0.11	28.93 ± 5.26
Cisplatin	6.50 ± 0.46	4.07 ± 0.18	3.71 ± 0.42	6.84 ± 0.61

[a] IC_{50}: concentration of the test compound that inhibits 50% of cell growth. Results are expressed as the mean ± SD (n = 3).

2.5. Effect of 15a on cell cycle

To clarify whether the inhibition of cell growth by **15a** is caused by a cell-cycle effect, the DNA content of cell nuclei was measured by flow cytometry (Figure 4). Incubation of HepG2 cells with **15a** at concentrations of 0.5, 1 and 2 μM resulted in accumulation of 23.38%, 29.57% and 41.45% of cells at the G2 phase, respectively, as compared with 17.45% in the control group. Meanwhile, the percentage of cells at the G1 phase decreased to 51.14%, 47.33% and 34.32%, respectively, as compared with 56.77% in the control group. These results indicated that **15a** inhibited the growth of HepG2 cells by inducing G2-phase arrest of the cell cycle.

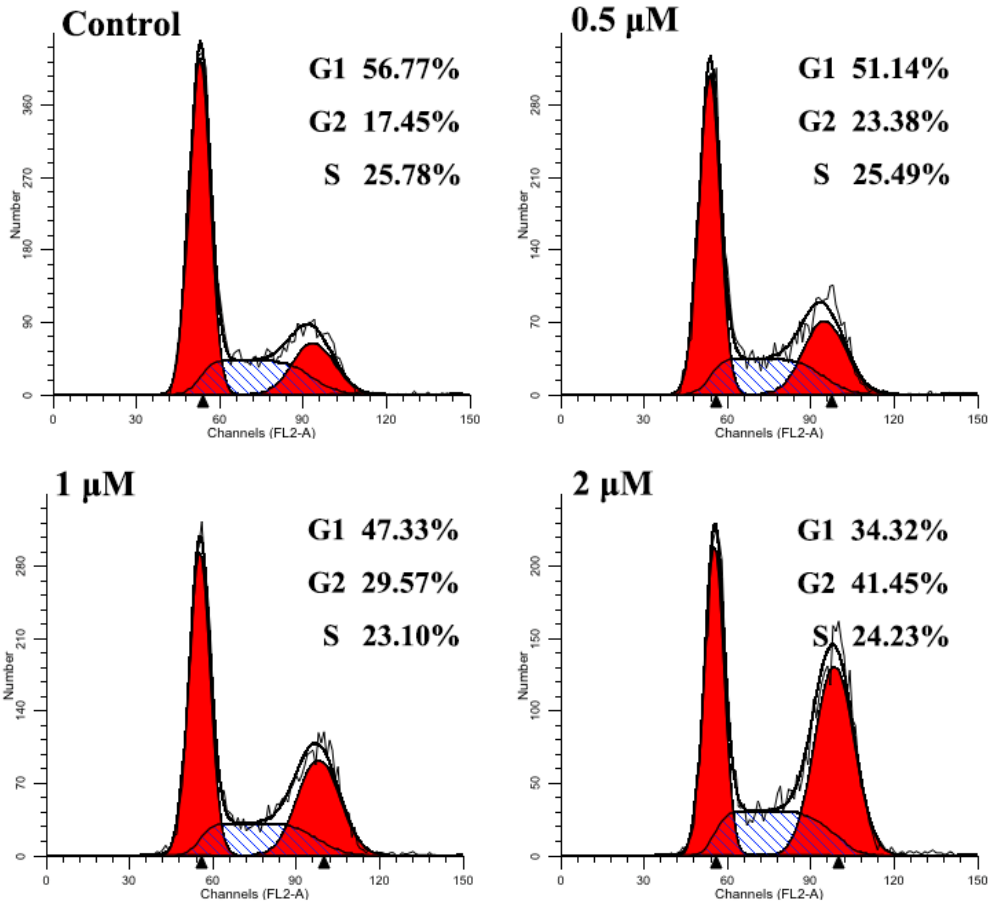


Figure 4. Effect of **15a** on cell cycle progression of HepG2. HepG2 cells were treated with varying concentrations of **15a** (0, 0.5, 1, 2 μM) for 72 h and stained with propidium iodide (PI). Cellular DNA content for cell-cycle distribution analysis was measured by flow cytometry.

2.6. Effect of 15a on cell apoptosis

To verify whether the decrease of cancer cell viability is related with cell apoptosis induced by **15a**, an Annexin V-APC/7-AAD binding assay was carried out. As shown in Figure 5, **15a** induced apoptosis of HepG2 cells in a dose-dependent manner. HepG2 cells were treated with **15a** at concentrations of 0.5, 1 and 2 μM for 72 h, which resulted in 13.41%, 32.08% and 51.14% apoptotic cells (Q2 + Q4), as compared with 2.57% in the control group, indicating that **15a** induced apoptotic cell death in HepG2 cells.

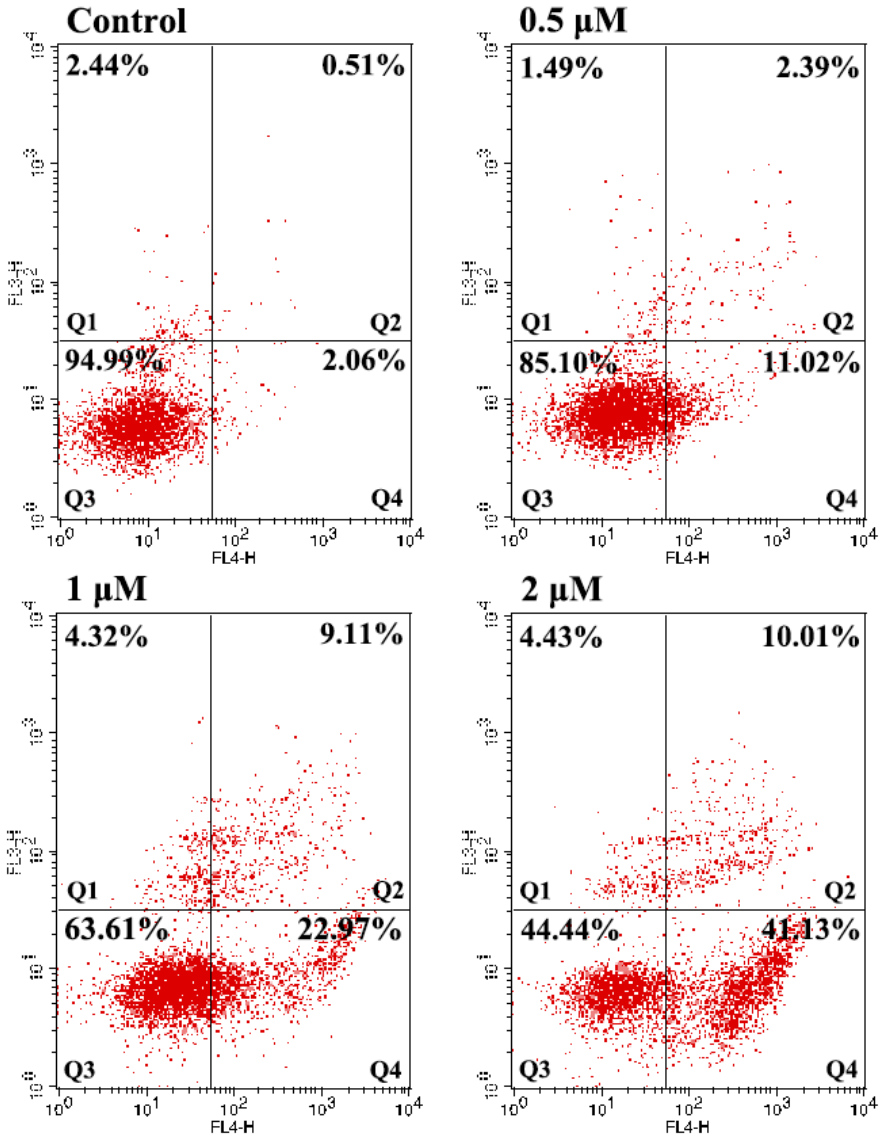


Figure 5. Effect of **15a** on apoptosis of HepG2 cells. Treatment with **15a** (0, 0.5, 1, 2 μM) for 72 h, HepG2 cells were collected and stained with Annexin V-APC/7-AAD, followed by flow cytometric analysis.

2.7. Effect of 15a on mitochondrial membrane potential

It is well known that mitochondria plays a key role in the process of apoptosis [31]. In order to further examine whether **15a**-induced cell apoptosis was involved in the disruption of mitochondrial membrane integrity, the lipophilic mitochondrial probe JC-1 was employed to measure the mitochondrial membrane potential (MMP). Treated with **15a** at concentrations of 0.5, 1 and 2 μM for 72 h, the number of HepG2 cells with collapsed MMP increased to 12.06%, 25.52% and 40.52%, respectively, as compared with 3.05% in the control group (Figure 6). These results suggested that **15a** caused mitochondrial depolarization of HepG2 cells in the process of apoptosis.

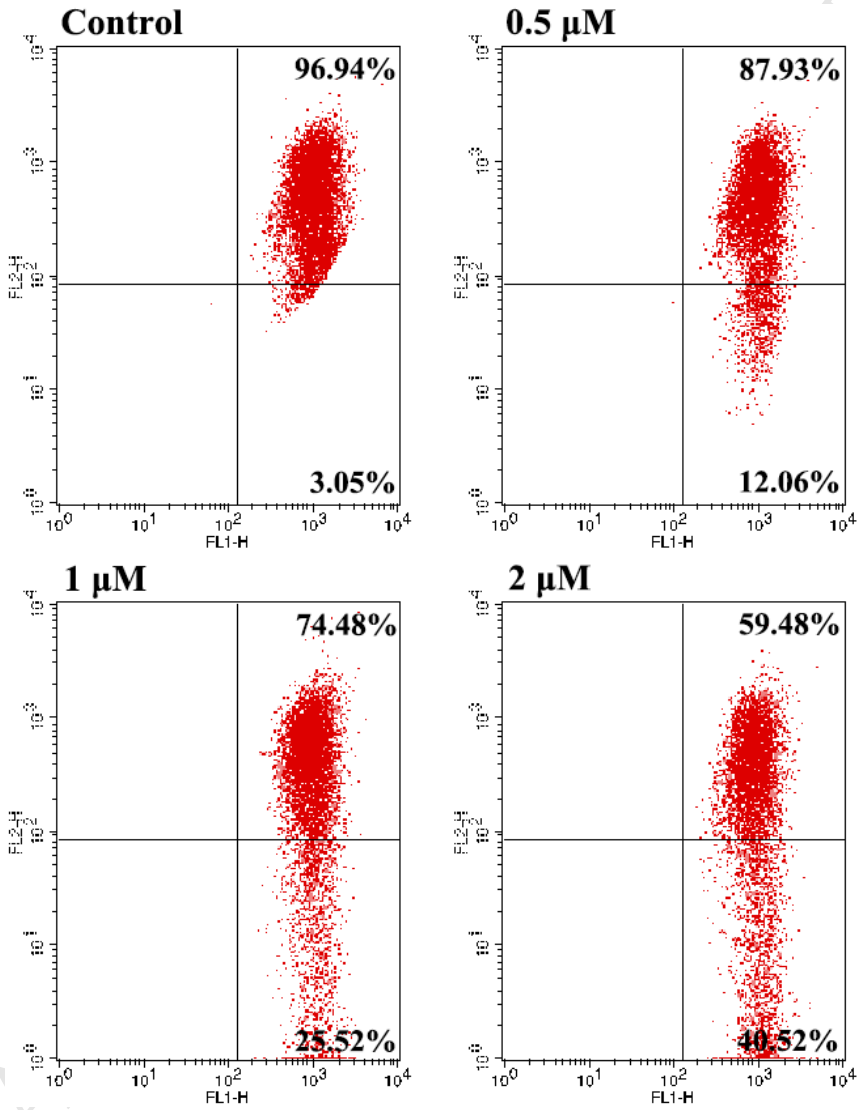


Figure 6. Effect of **15a** on the mitochondrial membrane potentials of HepG2 cells. Incubation with different concentrations (0, 0.5, 1, 2 μM) of **15a** in HepG2 cells for 72 h prior to staining with JC-1 dye, the number of cells with collapsed mitochondrial membrane potentials was determined by flow cytometry analysis.

2.8. *In vivo* anticancer activity of 15a

Based on the *in vitro* antiproliferative activity and mechanistic studies, we further tested the *in vivo* antitumor activity of **15a**. Human liver cancer xenograft was established by subcutaneous

inoculation of H22 cells into the right flank of mice. Forty mice were then randomly divided into five groups and treated intravenously with 30 mg/kg palmatine**,** 15 or 30 mg/kg **15a** and 20 mg/kg cyclophosphamide once a day, respectively. All the mice were sacrificed after 21 days, and the tumors were excised and weighed. The results were presented in Table 3. Treatment with **15a** at doses of 15 and 30 mg/kg resulted in tumor inhibitory rates (TIR) of 45.9% and 62.5%, respectively, which was significantly superior to the parent compound palmatine with a TIR of 41.6% at a dose of 30 mg/kg. Thus, **15a** may deserve further investigation as a potential anti-HCC drug candidate.

Table 3. *In vivo* antitumor activity of **15a** against mice bearing H22 liver cancer

Drugs	Dose mg/kg	Number of mice		Weight of mice (g)		Weight of tumor X ± SD (g)	Ratio of inhibition (%)
		Start	End	Start	End		
Control[a]	-	8	8	18.2 ± 0.3	26.0 ± 0.6	4.38 ± 0.16	/
Palmatine	30	8	8	18.1 ± 0.2	25.5 ± 0.8	2.56 ± 0.20**	41.6%
15a	15	8	8	18.2 ± 0.1	25.4 ± 0.4	2.37 ± 0.19**	45.9%
	30	8	8	18.3 ± 0.3	25.2 ± 0.6	1.64 ± 0.16**	62.5%
CP[b]	20	8	8	18.2 ± 0.2	25.0 ± 0.5	1.44 ± 0.12**	67.1%

[a] Control: vehicle of 10% DMF/2% Tween 80/88% saline.

[b] CP: cyclophosphamide. ** $P < 0.01$ *vs.* control group.

3. Conclusion

In summary, a series of novel NO-donating protoberberine derivatives were synthesized and their *in vitro* anti-HCC activities were initially evaluated. The bioassay results indicated that most of the derivatives exhibited significantly improved antiproliferative activity against HepG2 cells compared to parent compounds berberine and palmatine. Among them, compound **15a** displayed the most potent activity, which was superior to that of positive control cisplatin. Moreover, most compounds released moderate levels of NO *in vitro*, and the antitumor activity of **15a** in HepG2 cells was significantly attenuated by an NO scavenger in a dose-dependent manner. Interestingly, compound **15a** showed a broad-spectrum antitumor activity and possessed selective cytotoxicity between tumor cells (HepG2, SMMC-7721, HCT-116, HL-60) and normal liver LO-2 cells. Further mechanism studies revealed that **15a** caused G2 phase arrest of the cell cycle and induced apoptosis of HepG2 cells by mitochondrial depolarization. Finally, the *in vivo* antitumor activity of **15a** was validated in H22 liver cancer xenograft mouse model. Altogether, the remarkable biological profiles of these novel NO-releasing protoberberine derivatives may make them promising candidates for human cancer prevention.

4. Experimental section

4.1. Chemistry

4.1.1. General

Most chemicals and solvents were purchased from commercial sources. Further purification and drying by standard methods were employed when necessary. Melting points (m.p.) were taken on an XT-4 micro melting point apparatus and uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker-300 spectrometers in the indicated solvents (TMS as internal standard). Data

are reported as follows: chemical shift in ppm (δ), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, brs = broad singlet, m = multiplet), coupling constant (Hz), and integration. High Resolution Mass measurement was performed on Agilent QTOF 6520 mass spectrometer with electron spray ionization (ESI) as the ion source. Purity of all tested compounds was ≥ 95%, as estimated by HPLC analysis. Flash column chromatography was carried out using commercially available silica gel (200-300 mesh) under pressure. Intermediates **5** and **9** were synthesized according to the reported procedure [29,32,33].

4.1.2. General procedure for synthesis of the title compound 8

4.1.2.1. Synthesis of intermediate 6

To a solution of **5** (260 mg, 0.8 mmol), DCC (181 mg, 0.88 mmol) and DMAP (10 mg, 0.08 mmol) in CH_2Cl_2 (10 mL) was added the corresponding bromoalkane acid (1.2 mmol), the mixture was stirred for 4-8 h at room temperature. The reaction mixture was then diluted with CH_2Cl_2, washed with water, and brine successively, dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by flash column chromatography using petroleum ether/ethyl acetate (4/1, V/V) as an eluent to afford the intermediate.

4.1.2.1.1. Intermediate 6a

Yellowish liquid, yield 84%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.01 (d, *J* = 8.4 Hz, 1H), 6.83 (d, *J* = 8.4 Hz, 1H), 6.72 (s, 1H), 6.59 (s, 1H), 5.92 (s, 2H), 4.00 (d, *J* = 15.4 Hz, 1H), 3.80 (s, 3H), 3.58 – 3.55 (m, 1H), 3.47 – 3.41 (m, 3H), 3.23 (dd, *J* = 15.9, 3.2 Hz, 1H), 3.13 – 3.04 (m, 2H), 2.87 – 2.78 (m, 1H), 2.70 – 2.54 (m, 4H), 1.99 – 1.88 (m, 2H), 1.88 – 1.77 (m, 2H), 168 – 1.55 (m, 2H); MS (ESI) *m/z*: 502.11 $[M+H]^+$.

4.1.2.1.2. Intermediate 6b

Yellowish liquid, yield 87%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.01 (d, *J* = 8.4 Hz, 1H), 6.83 (d, *J* = 8.4 Hz, 1H), 6.72 (s, 1H), 6.59 (s, 1H), 5.92 (s, 2H), 4.00 (d, *J* = 15.5 Hz, 1H), 3.80 (s, 3H), 3.59 – 3.53 (m, 1H), 3.46 – 3.40 (m, 3H), 3.22 (dd, *J* = 15.9, 3.4 Hz, 1H), 3.13 – 3.04 (m, 2H), 2.87 – 2.78 (m, 1H), 2.67 – 2.55 (m, 4H), 1.94 – 1.73 (m, 4H), 1.54 – 1.35 (m, 6H); MS (ESI) *m/z*: 530.13 $[M+H]^+$.

4.1.2.1.3. Intermediate 6c

Yellowish liquid, yield 85%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.00 (d, *J* = 8.4 Hz, 1H), 6.82 (d, *J* = 8.5 Hz, 1H), 6.72 (s, 1H), 6.59 (s, 1H), 5.92 (s, 2H), 4.00 (d, *J* = 15.6 Hz, 1H), 3.80 (s, 3H), 3.58 – 3.54 (m, 1H), 3.46 – 3.39 (m, 3H), 3.22 (dd, *J* = 15.9, 3.4 Hz, 1H), 3.13 – 3.04 (m, 2H), 2.87 – 2.78 (m, 1H), 2.67 – 2.55 (m, 4H), 1.92 – 1.71 (m, 4H), 1.49 – 1.24 (m, 10H); MS (ESI) *m/z*: 558.17 $[M+H]^+$.

4.1.2.2. Synthesis of intermediate 7

6 (0.6 mmol) and $AgNO_3$ (204 mg, 1.2 mmol) was added in dry acetonitrile (10 mL), and stirred at 85 ^{o}C for 2-4 h. The reaction mixture was filtered and concentrated, and the residue was purified by silica gel column chromatography using petroleum ether/ethyl acetate (4/1, V/V) as an eluent to give the intermediate.

4.1.2.2.1. Intermediate 7a

Yellowish liquid, yield 84%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.00 (d, *J* = 8.4 Hz, 1H), 6.82 (d, *J* = 8.4 Hz, 1H), 6.71 (s, 1H), 6.58 (s, 1H), 5.90 (s, 2H), 4.47 (t, *J* = 6.5 Hz, 2H), 3.98 (d, *J* = 15.5 Hz, 1H), 3.79 (s, 3H), 3.58 – 3.51 (m, 1H), 3.42 (d, *J* = 15.4 Hz, 1H), 3.21 (dd, *J* = 15.9, 3.3 Hz, 1H), 3.12 – 3.02 (m, 2H), 2.85 – 2.76 (m, 1H), 2.68 – 2.54 (m, 4H), 1.89 – 1.74 (m, 4H), 1.64 – 1.51 (m, 2H); MS (ESI) *m/z*: 485.14 $[M+H]^+$.

4.1.2.2.2. Intermediate 7b

Yellowish liquid, yield 82%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.00 (d, *J* = 8.4 Hz, 1H), 6.82 (d, *J* = 8.4 Hz, 1H), 6.72 (s, 1H), 6.58 (s, 1H), 5.91 (s, 2H), 4.45 (t, *J* = 6.6 Hz, 2H), 3.99 (d, *J* = 15.5 Hz, 1H), 3.79 (s, 3H), 3.59 – 3.52 (m, 1H), 3.43 (d, *J* = 15.4 Hz, 1H), 3.22 (dd, *J* = 15.9, 3.3 Hz, 1H), 3.12 – 3.03 (m, 2H), 2.86 – 2.77 (m, 1H), 2.67 – 2.54 (m, 4H), 1.87 – 1.68 (m, 4H), 1.43 (s, 6H); MS (ESI) *m/z*: 513.19 $[M+H]^+$.

4.1.2.2.3. Intermediate 7c

Yellowish liquid, yield 83%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.00 (d, *J* = 8.4 Hz, 1H), 6.82 (d, *J* = 8.4 Hz, 1H), 6.72 (s, 1H), 6.58 (s, 1H), 5.91 (s, 2H), 4.44 (t, *J* = 6.7 Hz, 2H), 4.00 (d, *J* = 15.5 Hz, 1H), 3.79 (s, 3H), 3.57 – 3.53 (m, 1H), 3.43 (d, *J* = 15.4 Hz, 1H), 3.22 (dd, *J* = 15.9, 3.4 Hz, 1H), 3.16 – 3.03 (m, 2H), 2.86 – 2.77 (m, 1H), 2.67 – 2.54 (m, 4H), 1.84 – 1.64 (m, 4H), 1.51 – 1.24 (m, 10H); MS (ESI) *m/z*: 541.23 $[M+H]^+$.

4.1.2.3. Synthesis of the title compound 8

7 (0.4 mmol) and NBS (107 mg, 0.6 mmol) was added in $CHCl_3$ (5 mL), and stirred at 60 ^{o}C for 1 h. The reaction mixture was cooled to room temperature and then filtered. The residue was recrystallized in ethanol to give the product.

4.1.2.3.1. Intermediate 8a

Yellow solid, yield 65%. m.p. 158 – 160 ^{o}C; ^{1}H NMR (300 MHz, DMSO) δ 9.86 (s, 1H), 9.03 (s, 1H), 8.30 (d, *J* = 9.3 Hz, 1H), 8.20 (d, *J* = 9.2 Hz, 1H), 7.82 (s, 1H), 7.11 (s, 1H), 6.19 (s, 2H), 5.01 – 4.85 (m, 2H), 4.58 (t, *J* = 6.4 Hz, 2H), 4.04 (s, 3H), 3.29 – 3.14 (m, 2H), 2.88 (t, *J* = 7.2 Hz, 2H), 1.84 – 1.71 (m, 4H), 1.59 – 1.47 (m, 2H); ^{13}C NMR (75 MHz, DMSO) δ 170.45, 150.30, 149.94, 147.66, 144.29, 138.04, 133.42, 132.85, 130.76, 126.62, 125.81, 121.07, 120.51, 120.28, 108.38, 105.45, 102.11, 73.69, 57.15, 55.34, 32.94, 26.13, 25.77, 24.48, 23.77; HRMS (ESI) calculated for $C_{25}H_{25}N_2O_8$ $[M-Br]^+$ 481.1605, found 481.1607.

4.1.2.3.2. Intermediate 8b

Yellow solid, yield 54%. m.p. 155 – 157 ^{o}C; ^{1}H NMR (300 MHz, DMSO) δ 9.86 (s, 1H), 9.03 (s, 1H), 8.30 (d, *J* = 9.2 Hz, 1H), 8.20 (d, *J* = 9.3 Hz, 1H), 7.82 (s, 1H), 7.11 (s, 1H), 6.19 (s, 2H), 5.00 – 4.85 (m, 2H), 4.54 (t, *J* = 6.6 Hz, 2H), 4.03 (s, 3H), 3.28 – 3.19 (m, 2H), 2.86 (t, *J* = 7.3 Hz, 2H), 1.81 – 1.65 (m, 4H), 1.41 (s, 6H); ^{13}C NMR (75 MHz, DMSO) δ 170.57, 150.32, 149.94, 147.66, 144.31, 138.04, 133.51, 132.89, 130.78, 126.61, 125.85, 121.10, 120.53, 120.30, 108.38, 105.48, 102.10, 73.83, 57.17, 55.29, 33.12, 28.17, 28.08, 26.13, 25.96, 24.94, 24.09; HRMS (ESI) calculated for $C_{27}H_{29}N_2O_8$ $[M-Br]^+$ 509.1918, found 509.1928.

4.1.2.3.3. Intermediate 8c

Yellow solid, yield 57%. m.p. 154 – 156 ^{o}C; ^{1}H NMR (300 MHz, DMSO) δ 9.85 (s, 1H), 9.03 (s, 1H), 8.29 (d, *J* = 9.3 Hz, 1H), 8.20 (d, *J* = 9.1 Hz, 1H), 7.82 (s, 1H), 7.11 (s, 1H), 6.19 (s, 2H), 4.99 – 4.85 (m, 2H), 4.52 (t, *J* = 6.5 Hz, 2H), 4.03 (s, 3H), 3.29 – 3.16 (m, 2H), 2.85 (t, *J* = 7.3 Hz, 2H), 1.80 – 1.62 (m, 4H), 1.45 – 1.23 (m, 10H); ^{13}C NMR (75 MHz, DMSO) δ 170.55, 150.32, 149.95, 147.68, 144.30, 138.05, 133.51, 132.87, 130.76, 126.59, 125.85, 121.09, 120.51, 120.29, 108.38, 105.46, 102.11, 73.83, 57.15, 55.33, 33.13, 28.70, 28.59, 28.46, 28.26, 26.14, 25.98, 25.03, 24.21; HRMS (ESI) calculated for $C_{29}H_{33}N_2O_8$ $[M-Br]^+$ 537.2231, found 537.2236.

4.1.3. General procedure for synthesis of the target compound 12

4.1.3.1. Synthesis of intermediate 10

To a solution of **9** (1 mmol) in methanol (10 mL) was added sodium borohydride (114 mg, 3 mmol), the mixture was stirred for 1 h at room temperature. The reaction mixture was

concentrated and then dissolved in CH_2Cl_2 (15 mL), washed with water, brine, dried over anhydrous Na_2SO_4 successively. The organic layer was concentrated and purified by column chromatography on silica gel using petroleum ether/ethyl acetate (8/1, V/V) as an eluent to provide the intermediate.

4.1.3.1.1. Intermediate 10a

Yellowish liquid, yield 78%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.88 (d, *J* = 8.4 Hz, 1H), 6.78 (d, *J* = 8.4 Hz, 1H), 6.73 (s, 1H), 6.60 (s, 1H), 5.92 (s, 2H), 4.44 – 4.23 (m, 3H), 3.84 (s, 3H), 3.65 (t, *J* = 6.3 Hz, 2H), 3.61 – 3.48 (m, 2H), 3.28 – 3.04 (m, 3H), 2.89 – 2.74 (m, 1H), 2.69 – 2.60 (m, 2H); MS (ESI) *m/z*: 432.07 $[M+H]^+$.

4.1.3.1.2. Intermediate 10b

Yellowish liquid, yield 81%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.87 (d, *J* = 8.4 Hz, 1H), 6.78 (d, *J* = 8.4 Hz, 1H), 6.73 (s, 1H), 6.59 (s, 1H), 5.92 (s, 2H), 4.26 (d, *J* = 15.7 Hz, 1H), 4.18 – 4.03 (m, 2H), 3.83 (s, 3H), 3.71 (t, *J* = 6.5 Hz, 2H), 3.57 – 3.52 (m, 2H), 3.26 – 3.06 (m, 3H), 2.85 – 2.76 (m, 1H), 2.68 – 2.59 (m, 2H), 2.42 – 2.23 (m, 2H); MS (ESI) *m/z*: 446.08 $[M+H]^+$.

4.1.3.1.3. Intermediate 10c

Yellowish liquid, yield 73%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.86 (d, *J* = 8.3 Hz, 1H), 6.77 (d, *J* = 8.4 Hz, 1H), 6.73 (s, 1H), 6.59 (s, 1H), 5.92 (s, 2H), 4.21 (d, *J* = 15.7 Hz, 1H), 4.08 – 3.93 (m, 2H), 3.83 (s, 3H), 3.56 – 3.50 (m, 4H), 3.25 – 3.06 (m, 3H), 2.85 – 2.76 (m, 1H), 2.69 – 2.57 (m, 2H), 2.17 – 2.07 (m, 2H), 1.97 – 1.88 (m, 2H); MS (ESI) *m/z*: 460.09 $[M+H]^+$.

4.1.3.1.4. Intermediate 10d

Yellowish liquid, yield 75%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.86 (d, *J* = 8.4 Hz, 1H), 6.78 (d, *J* = 8.4 Hz, 1H), 6.73 (s, 1H), 6.60 (s, 1H), 5.92 (s, 2H), 4.23 (d, *J* = 15.7 Hz, 1H), 4.06 – 3.91 (m, 2H), 3.83 (s, 3H), 3.59 – 3.55 (m, 2H), 3.46 (t, *J* = 6.8 Hz, 2H), 3.25 – 3.06 (m, 3H), 2.85 – 2.76 (m, 1H), 2.71 – 2.57 (m, 2H), 2.00 – 1.91 (m, 2H), 1.87 – 1.76 (m, 2H), 1.70 – 1.65 (m, 2H); MS (ESI) *m/z*: 474.60 $[M+H]^+$.

4.1.3.1.5. Intermediate 10e

Yellowish liquid, yield 74%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.85 (d, *J* = 8.4 Hz, 1H), 6.77 (d, *J* = 8.4 Hz, 1H), 6.73 (s, 1H), 6.59 (s, 1H), 5.92 (s, 2H), 4.23 (d, *J* = 15.8 Hz, 1H), 4.05 – 3.90 (m, 2H), 3.83 (s, 3H), 3.55 – 3.50 (m, 2H), 3.44 (t, *J* = 6.8 Hz, 2H), 3.25 – 3.07 (m, 3H), 2.86 – 2.77 (m, 1H), 2.68 – 2.58 (m, 2H), 1.98 – 1.87 (m, 2H), 1.83 – 1.76 (m, 2H), 1.56 – 1.50 (m, 4H); MS (ESI) *m/z*: 488.61 $[M+H]^+$.

4.1.3.1.6. Intermediate 10f

Yellowish liquid, yield 77%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.85 (d, *J* = 8.4 Hz, 1H), 6.77 (d, *J* = 8.4 Hz, 1H), 6.73 (s, 1H), 6.60 (s, 1H), 5.92 (s, 2H), 5.30 (s, 1H), 4.23 (d, *J* = 15.8 Hz, 1H), 4.04 – 3.89 (m, 2H), 3.83 (s, 3H), 3.55 – 3.50 (m, 2H), 3.42 (t, *J* = 6.8 Hz, 2H), 3.25 – 3.07 (m, 3H), 2.86 – 2.77 (m, 1H), 2.68 – 2.58 (m, 2H), 1.92 – 1.82 (m, 2H), 1.81 – 1.73 (m, 2H), 1.48 – 1.43 (m, 4H), 1.41 – 1.38 (m, 4H); MS (ESI) *m/z*: 516.17 $[M+H]^+$.

4.1.3.1.7. Intermediate 10g

Yellowish liquid, yield 71%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.85 (d, *J* = 8.4 Hz, 1H), 6.78 (d, *J* = 8.4 Hz, 1H), 6.73 (s, 1H), 6.60 (s, 1H), 5.92 (s, 2H), 4.23 (d, *J* = 15.8 Hz, 1H), 4.06 – 3.89 (m, 2H), 3.83 (s, 3H), 3.55 – 3.50 (m, 2H), 3.41 (t, *J* = 6.8 Hz, 2H), 3.25 – 3.06 (m, 3H), 2.85 – 2.76 (m, 1H), 2.68 – 2.57 (m, 2H), 1.90 – 1.81 (m, 2H), 1.80 – 1.72 (m, 2H), 1.51 – 1.23 (m, 12H); MS (ESI) *m/z*: 544.16 $[M+H]^+$.

4.1.3.1.8. Intermediate 10h

Yellowish liquid, yield 73%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.85 (d, *J* = 8.3 Hz, 1H), 6.77 (d, *J* = 8.4 Hz, 1H), 6.73 (s, 1H), 6.59 (s, 1H), 5.92 (s, 2H), 4.24 (d, *J* = 15.8 Hz, 1H), 4.05 – 3.89 (m, 2H), 3.83 (s, 3H), 3.55 – 3.50 (m, 2H), 3.41 (t, *J* = 6.9 Hz, 2H), 3.25 – 3.06 (m, 3H), 2.86 – 2.77 (m, 1H), 2.68 – 2.59 (m, 2H), 1.90 – 1.80 (m, 2H), 1.79 – 1.72 (m, 2H), 1.52 – 1.22 (m, 16H); MS (ESI) *m/z*: 572.22 $[M+H]^+$.

4.3.2. Synthesis of intermediate 11

Intermediate **11** was prepared by the same method as intermediate **7**

4.1.3.2.1. Intermediate 11a

Yellowish liquid, yield 82%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.88 (d, *J* = 8.4 Hz, 1H), 6.77 (d, *J* = 8.4 Hz, 1H), 6.71 (s, 1H), 6.59 (s, 1H), 5.91 (s, 2H), 4.82 – 4.71 (m, 2H), 4.39 – 4.32 (m, 1H), 4.19 – 4.27 (m, 2H), 3.83 (d, *J* = 6.9 Hz, 3H), 3.51 – 3.46 (m, 2H), 3.24 – 3.05 (m, 3H), 2.84 – 2.75 (m, 1H), 2.68 – 2.58 (m, 2H); MS (ESI) *m/z*: 415.12 $[M+H]^+$.

4.1.3.2.2. Intermediate 11b

Yellowish liquid, yield 83%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.87 (d, *J* = 8.4 Hz, 1H), 6.77 (d, *J* = 8.4 Hz, 1H), 6.72 (s, 1H), 6.59 (s, 1H), 5.91 (s, 2H), 4.77 (t, *J* = 6.3 Hz, 2H), 4.18 (d, *J* = 15.6 Hz, 1H), 4.21 – 4.00 (m, 2H), 3.81 (s, 3H), 3.53 – 3.48 (m, 2H), 3.24 – 3.05 (m, 3H), 2.84 – 2.75 (m, 1H), 2.67 – 2.56 (m, 2H), 2.22 – 2.14 (m, 2H); MS (ESI) *m/z*: 429.16 $[M+H]^+$.

4.1.3.2.3. Intermediate 11c

Yellowish liquid, yield 86%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.85 (d, *J* = 8.4 Hz, 1H), 6.77 (d, *J* = 8.4 Hz, 1H), 6.72 (s, 1H), 6.58 (s, 1H), 5.90 (s, 2H), 4.58 (t, *J* = 6.4 Hz, 2H), 4.19 (d, *J* = 15.7 Hz, 1H), 4.07 – 3.93 (m, 2H), 3.81 (s, 3H), 3.53 – 3.48 (m, 2H), 3.24 – 3.05 (m, 3H), 2.84 – 2.75 (m, 1H), 2.67 – 2.56 (m, 2H), 2.05 – 1.94 (m, 2H), 1.93 – 1.81 (m, 2H); MS (ESI) *m/z*: 443.14 $[M+H]^+$.

4.1.3.2.4. Intermediate 11d

Yellowish liquid, yield 82%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.85 (d, *J* = 8.4 Hz, 1H), 6.77 (d, *J* = 8.4 Hz, 1H), 6.72 (s, 1H), 6.58 (s, 1H), 5.90 (s, 2H), 4.49 (t, *J* = 6.6 Hz, 2H), 4.21 (d, *J* = 15.7 Hz, 1H), 4.05 – 3.91 (m, 2H), 3.82 (s, 3H), 3.53 – 3.48 (m, 2H), 3.24 – 3.05 (m, 3H), 2.84 – 2.75 (m, 1H), 2.67 – 2.56 (m, 2H), 1.87 – 1.77 (m, 4H), 1.67 – 1.58 (m, 2H); MS (ESI) *m/z*: 457.19 $[M+H]^+$.

4.1.3.2.5. Intermediate 11e

Yellowish liquid, yield 87%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.85 (d, *J* = 8.3 Hz, 1H), 6.77 (d, *J* = 8.4 Hz, 1H), 6.72 (s, 1H), 6.59 (s, 1H), 5.90 (s, 2H), 4.46 (t, *J* = 6.6 Hz, 2H), 4.21 (d, *J* = 15.7 Hz, 1H), 4.05 – 3.89 (m, 2H), 3.82 (s, 3H), 3.54 – 3.49 (m, 2H), 3.24 – 3.06 (m, 3H), 2.85 – 2.76 (m, 1H), 2.67 – 2.56 (m, 2H), 1.84 – 1.68 (m, 4H), 1.60 – 1.43 (m, 4H); MS (ESI) *m/z*: 471.19 $[M+H]^+$.

4.1.3.2.6. Intermediate 11f

Yellowish liquid, yield 85%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.84 (d, *J* = 8.3 Hz, 1H), 6.77 (d, *J* = 8.4 Hz, 1H), 6.72 (s, 1H), 6.58 (s, 1H), 5.90 (s, 2H), 4.44 (t, *J* = 6.7 Hz, 2H), 4.22 (d, *J* = 15.8 Hz, 1H), 4.04 – 3.89 (m, 2H), 3.81 (s, 3H), 3.54 – 3.49 (m, 2H), 3.24 – 3.06 (m, 3H), 2.85 – 2.76 (m, 1H), 2.67 – 2.56 (m, 2H), 1.79 – 1.68 (m, 4H), 1.50 – 1.34 (m, 8H); MS (ESI) *m/z*: 499.17 $[M+H]^+$.

4.1.3.2.7. Intermediate 11g

Yellowish liquid, yield 81%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.84 (d, *J* = 8.4 Hz, 1H), 6.77 (d, *J* = 8.4 Hz, 1H), 6.73 (s, 1H), 6.59 (s, 1H), 5.90 (s, 2H), 4.43 (t, *J* = 6.7 Hz, 2H), 4.23 (d, *J* = 15.8

Hz, 1H), 4.05 – 3.89 (m, 2H), 3.82 (s, 3H), 3.54 – 3.49 (m, 2H), 3.24 – 3.06 (m, 3H), 2.85 – 2.76 (m, 1H), 2.67 – 2.56 (m, 2H), 1.83 – 1.62 (m, 4H), 1.52 – 1.24 (m, 12H); MS (ESI) *m/z*: 527.27 $[M+H]^+$.

4.1.3.2.8. Intermediate 11h

Yellowish liquid, yield 82%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.84 (d, *J* = 8.4 Hz, 1H), 6.76 (d, *J* = 8.4 Hz, 1H), 6.72 (s, 1H), 6.58 (s, 1H), 5.90 (s, 2H), 4.42 (t, *J* = 6.7 Hz, 2H), 4.23 (d, *J* = 15.8 Hz, 1H), 4.05 – 3.89 (m, 2H), 3.81 (s, 3H), 3.54 – 3.49 (m, 2H), 3.24 – 3.06 (m, 3H), 2.84 – 2.75 (m, 1H), 2.67 – 2.56 (m, 2H), 1.84 – 1.62 (m, 4H), 1.53 – 1.21 (m, 16H); MS (ESI) *m/z*: 555.29 $[M+H]^+$.

4.1.3.3. Synthesis of the title compound 12

Compounds **12a-e** were prepared by the same method as compound **8.** Compounds **12f-h** were obtained by the following method: the reaction mixture was diluted with $CHCl_3$, washed with water, and brine successively, dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by column chromatography on silica gel using CH_2Cl_2/MeOH (40/1, V/V) as an eluent to give the products.

4.1.3.3.1. Compound 12a

Yellow solid, yield 65%. m.p. 233 – 235 °C; ^{1}H NMR (300 MHz, DMSO) ^{1}H NMR (300 MHz, DMSO) δ 9.88 (s, 1H), 8.96 (s, 1H), 8.22 (d, *J* = 9.2 Hz, 1H), 8.03 (d, *J* = 9.2 Hz, 1H), 7.81 (s, 1H), 7.11 (s, 1H), 6.18 (s, 2H), 5.05 – 4.98 (m, 2H), 4.98 – 4.87 (m, 2H), 4.64 – 4.56 (m, 2H), 4.06 (s, 3H), 3.28 – 3.14 (m, 2H); ^{13}C NMR (75 MHz, DMSO) δ 150.09, 149.78, 147.61, 145.32, 141.88, 137.42, 132.83, 130.57, 126.39, 123.86, 121.29, 120.31, 120.11, 108.38, 105.36, 102.06, 73.07, 69.86, 57.01, 55.26, 26.26; HRMS (ESI) calculated for $C_{21}H_{19}N_2O_7$ $[M-Br]^+$ 411.1187, found 411.1193.

4.1.3.3.2. Compound 12b

Yellow solid, yield 63%. m.p. 230 – 232 °C; ^{1}H NMR (300 MHz, DMSO) δ 9.83 (s, 1H), 8.96 (s, 1H), 8.21 (d, *J* = 9.2 Hz, 1H), 8.01 (d, *J* = 9.1 Hz, 1H), 7.81 (s, 1H), 7.10 (s, 1H), 6.18 (s, 2H), 4.95 (t, *J* = 6.0 Hz, 2H), 4.84 (t, *J* = 6.3 Hz, 2H), 4.39 (t, *J* = 6.1 Hz, 2H), 4.05 (s, 3H), 3.22 (t, *J* = 5.9 Hz, 2H), 2.35 – 2.27 (m, 2H); ^{13}C NMR (75 MHz, DMSO) δ 150.16, 149.73, 147.58, 145.31, 142.29, 137.33, 132.88, 130.57, 126.42, 123.52, 121.38, 120.33, 120.10, 108.35, 105.35, 102.05, 71.07, 70.61, 56.98, 55.19, 27.03, 26.24; HRMS (ESI) calculated for $C_{22}H_{21}N_2O_7$ $[M-Br]^+$ 425.1343, found 425.1354.

4.1.3.3.3. Compound 12c

Yellow solid, yield 61%. m.p. 227 – 229 °C; ^{1}H NMR (300 MHz, DMSO) δ 9.78 (s, 1H), 8.95 (s, 1H), 8.21 (d, *J* = 9.1 Hz, 1H), 8.00 (d, *J* = 9.2 Hz, 1H), 7.81 (s, 1H), 7.10 (s, 1H), 6.18 (s, 2H), 5.02 – 4.88 (m, 2H), 4.71 – 4.60 (m, 2H), 4.36 – 4.26 (m, 2H), 4.06 (s, 3H), 3.27 – 3.16 (m, 2H), 1.96 (s, 4H); ^{13}C NMR (75 MHz, DMSO) δ 150.32, 149.72, 147.57, 145.23, 142.54, 137.29, 132.89, 130.57, 126.43, 123.41, 121.48, 120.34, 120.12, 108.34, 105.34, 102.05, 73.63, 73.53, 56.96, 55.24, 26.26, 25.79, 22.81; HRMS (ESI) calculated for $C_{23}H_{23}N_2O_7$ $[M-Br]^+$ 439.1500, found 439.1505.

4.1.3.3.4. Compound 12d

Yellow solid, yield 64%. m.p. 222 – 224 °C; ^{1}H NMR (300 MHz, DMSO) δ 9.77 (s, 1H), 8.95 (s, 1H), 8.21 (d, *J* = 9.2 Hz, 1H), 8.00 (d, *J* = 9.1 Hz, 1H), 7.81 (s, 1H), 7.10 (s, 1H), 6.18 (s, 2H), 4.95 (t, *J* = 6.0 Hz, 2H), 4.59 (t, *J* = 6.5 Hz, 2H), 4.29 (t, *J* = 6.5 Hz, 2H), 4.06 (s, 3H), 3.21 (t, *J* = 5.9 Hz, 2H), 1.97 – 1.87 (m, 2H), 1.84 – 1.75 (m, 2H), 1.64 – 1.54 (m, 2H); ^{13}C NMR (75 MHz,

DMSO) δ 150.34, 149.71, 147.57, 145.21, 142.67, 137.29, 132.91, 130.56, 126.45, 123.32, 121.53, 120.36, 120.13, 108.34, 105.35, 102.04, 73.86, 73.80, 56.95, 55.25, 28.93, 26.25, 25.77, 21.58; HRMS (ESI) calculated for $C_{24}H_{25}N_2O_7$ $[M-Br]^+$ 453.1656, found 453.1657.

4.1.3.3.5. Compound 12e

Yellow solid, yield 58%. m.p. 219 – 221 °C; ^{1}H NMR (300 MHz, DMSO) δ 9.75 (s, 1H), 8.95 (s, 1H), 8.20 (d, *J* = 9.2 Hz, 1H), 7.99 (d, *J* = 9.1 Hz, 1H), 7.80 (s, 1H), 7.10 (s, 1H), 6.18 (s, 2H), 5.02 – 4.89 (m, 2H), 4.55 (t, *J* = 6.5 Hz, 2H), 4.28 (t, *J* = 6.6 Hz, 2H), 4.05 (s, 3H), 3.26 – 3.16 (m, 2H), 1.92 – 1.87 (m, 2H), 1.75 – 1.70 (m, 2H), 1.62 – 1.38 (m, 4H); ^{13}C NMR (75 MHz, DMSO) δ 150.33, 149.71, 147.57, 145.21, 142.72, 137.29, 132.92, 130.57, 126.48, 123.27, 121.54, 120.37, 120.13, 108.34, 105.36, 102.04, 74.00, 73.81, 56.96, 55.25, 29.23, 26.26, 25.98, 24.84, 24.80; HRMS (ESI) calculated for $C_{25}H_{27}N_2O_7$ $[M-Br]^+$ 467.1813, found 467.1817.

4.1.3.3.6. Compound 12f

Yellow solid, yield 71%. m.p. 211 – 213 °C; ^{1}H NMR (300 MHz, DMSO) δ 9.76 (s, 1H), 8.96 (s, 1H), 8.18 (d, *J* = 9.2 Hz, 1H), 7.99 (d, *J* = 9.1 Hz, 1H), 7.77 (s, 1H), 7.09 (s, 1H), 6.17 (s, 2H), 4.98 (s, 2H), 4.53 (t, *J* = 6.5 Hz, 2H), 4.27 (t, *J* = 6.6 Hz, 2H), 4.05 (s, 3H), 3.22 (s, 2H), 1.97 – 1.80 (m, 2H), 1.77 – 1.60 (m, 2H), 1.49 (s, 2H), 1.37 (s, 6H); ^{13}C NMR (75 MHz, DMSO) δ 150.34, 149.71, 147.58, 145.23, 142.76, 137.29, 132.92, 130.57, 126.50, 123.26, 121.56, 120.38, 120.14, 108.34, 105.36, 102.04, 74.16, 73.85, 56.96, 55.25, 29.41, 28.59, 28.49, 26.26, 25.98, 25.11, 25.02; HRMS (ESI) calculated for $C_{27}H_{31}N_2O_7$ $[M-Br]^+$ 495.2126, found 495.2133.

4.1.3.3.7. Compound 12g

Yellow solid, yield 67%. m.p. 206 – 208 °C; ^{1}H NMR (300 MHz, DMSO) δ 9.76 (s, 1H), 8.95 (s, 1H), 8.20 (d, *J* = 9.2 Hz, 1H), 7.99 (d, *J* = 9.1 Hz, 1H), 7.80 (s, 1H), 7.10 (s, 1H), 6.18 (s, 2H), 4.95 (t, *J* = 5.9 Hz, 2H), 4.51 (t, *J* = 6.6 Hz, 2H), 4.28 (t, *J* = 6.8 Hz, 2H), 4.05 (s, 3H), 3.21 (t, *J* = 5.9 Hz, 2H), 1.92 – 1.83 (m, 2H), 1.68 – 1.61 (m, 2H), 1.56 – 1.42 (m, 2H), 1.31 (s, 10H); ^{13}C NMR (75 MHz, DMSO) δ 150.34, 149.71, 147.58, 145.23, 142.76, 137.29, 132.93, 130.57, 126.50, 123.25, 121.56, 120.37, 120.14, 108.33, 105.36, 102.04, 74.18, 73.83, 56.96, 55.25, 29.45, 28.89, 28.78, 28.51, 26.27, 25.97, 25.21, 25.04; HRMS (ESI) calculated for $C_{29}H_{35}N_2O_7$ $[M-Br]^+$ 523.2439, found 523.2451.

4.1.3.3.8. Compound 12h

Yellow solid, yield 68%. m.p. 203 – 205 °C; ^{1}H NMR (300 MHz, DMSO) δ 9.76 (s, 1H), 8.95 (s, 1H), 8.20 (d, *J* = 9.1 Hz, 1H), 7.99 (d, *J* = 9.2 Hz, 1H), 7.81 (s, 1H), 7.10 (s, 1H), 6.18 (s, 2H), 4.95 (s, 2H), 4.50 (t, *J* = 6.5 Hz, 2H), 4.28 (t, *J* = 6.7 Hz, 2H), 4.05 (s, 3H), 3.21 (s, 2H), 1.95 – 1.76 (m, 2H), 1.70 – 1.55 (m, 2H), 1.48 (s, 2H), 1.27 (s, 14H); ^{13}C NMR (75 MHz, DMSO) δ 150.32, 149.70, 147.57, 145.22, 142.75, 137.26, 132.92, 130.54, 126.47, 123.24, 121.55, 120.36, 120.14, 108.32, 105.35, 102.04, 74.17, 73.81, 56.95, 55.24, 29.46, 29.01, 28.98, 28.91, 28.82, 28.51, 26.27, 25.97, 25.23, 25.03; HRMS (ESI) calculated for $C_{31}H_{39}N_2O_7$ $[M-Br]^+$ 551.2752, found 551.2759.

4.1.3.4. Synthesis of the title compound 13f

12f (0.2 mmol) and NBS (107 mg, 0.6 mmol) was added in $CHCl_3$ (5 mL), and stirred at 60 °C for 3 h. The reaction mixture was diluted with $CHCl_3$, washed with water, and brine successively, dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by column chromatography on silica gel using CH_2Cl_2/MeOH (80/1, V/V) as an eluent to afford the product as yellow solid, yield 44%. m.p. 184 – 186 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.50 (s, 1H), 8.17 (d, *J* = 9.3 Hz, 1H), 7.95 (d, *J* = 9.4 Hz, 1H), 7.81 (s, 1H), 6.87 (s, 1H), 6.12 (s, 2H), 5.29 (s, 2H),

4.56 (t, *J* = 6.8 Hz, 2H), 4.46 (t, *J* = 6.7 Hz, 2H), 4.09 (s, 3H), 3.25 (s, 2H), 2.12 – 1.98 (m, 2H), 1.80 – 1.66 (m, 2H), 1.55 (s, 2H), 1.41 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.33, 150.41, 147.16, 146.54, 145.57, 136.26, 134.26, 132.98, 126.20, 123.43, 121.55, 119.92, 119.13, 110.85, 108.21, 102.23, 75.83, 73.61, 58.10, 57.01, 30.15, 29.16, 29.02, 28.57, 26.69, 25.56, 25.53; HRMS (ESI) calculated for $C_{27}H_{30}BrN_2O_7$ $[M\text{-}Br]^+$ 573.1231, found 573.1230.

4.1.3.5. Synthesis of the title compound 14

Compound **14** was prepared from palmatine by the same method as compound **12.**

4.1.3.5.1. Compound 14a

Yellow solid, total yield 27%. m.p. 204 – 206 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.92 (s, 1H), 8.91 (s, 1H), 8.08 (d, *J* = 8.5 Hz, 1H), 7.64 (d, *J* = 8.6 Hz, 1H), 7.50 (s, 1H), 6.67 (s, 1H), 5.16 (s, 2H), 4.47 (t, *J* = 6.6 Hz, 2H), 4.37 (t, *J* = 6.6 Hz, 2H), 4.10 (s, 3H), 4.00 (s, 3H), 3.95 (s, 3H), 3.30 (s, 2H), 2.03 – 1.91 (m, 2H), 1.81 – 1.68 (m, 2H), 1.54 (s, 2H), 1.42 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.56, 149.97, 149.07, 144.76, 143.17, 137.38, 133.55, 127.49, 125.27, 123.87, 121.80, 120.76, 118.71, 110.26, 108.60, 74.86, 73.49, 57.10, 56.65, 56.25, 56.07, 30.05, 29.10, 28.97, 27.05, 26.62, 25.61, 25.52; HRMS (ESI) calculated for $C_{28}H_{35}N_2O_7$ $[M\text{-}Br]^+$ 511.2439, found 511.2449.

4.1.3.5.2. Compound 14b

Yellow solid, total yield 22%. m.p. 199 – 201 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.90 (s, 1H), 9.00 (s, 1H), 8.11 (d, *J* = 9.1 Hz, 1H), 7.62 (d, *J* = 9.0 Hz, 1H), 7.53 (s, 1H), 6.66 (s, 1H), 5.17 (s, 2H), 4.46 (t, *J* = 6.7 Hz, 2H), 4.38 (t, *J* = 6.8 Hz, 2H), 4.11 (s, 3H), 4.00 (s, 3H), 3.95 (s, 3H), 3.36 – 3.27 (m, 2H), 2.03 – 1.90 (m, 2H), 1.79 – 1.67 (m, 2H), 1.52 (s, 2H), 1.34 (s, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.48, 149.88, 149.01, 144.53, 143.04, 137.30, 133.55, 127.35, 125.16, 123.90, 121.74, 120.87, 118.68, 110.19, 108.64, 74.85, 73.47, 57.14, 56.59, 56.27, 56.02, 30.07, 29.35, 29.27, 29.24, 28.96, 27.02, 26.59, 25.70, 25.51; HRMS (ESI) calculated for $C_{30}H_{39}N_2O_7$ $[M\text{-}Br]^+$ 539.2752, found 539.2762.

4.1.3.5.3. Compound 14c

Yellow solid, total yield 24%. m.p. 197 – 199 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.87 (s, 1H), 9.01 (s, 1H), 8.12 (d, *J* = 8.2 Hz, 1H), 7.62 (d, *J* = 8.7 Hz, 1H), 7.53 (s, 1H), 6.66 (s, 1H), 5.15 (s, 2H), 4.45 (t, *J* = 6.7 Hz, 2H), 4.37 (t, *J* = 6.7 Hz, 2H), 4.11 (s, 3H), 4.00 (s, 3H), 3.94 (s, 3H), 3.32 (s, 2H), 2.03 – 1.89 (m, 2H), 1.78 – 1.66 (m, 2H), 1.51 (s, 2H), 1.29 (s, 14H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.45, 149.85, 148.98, 144.44, 142.98, 137.28, 133.52, 127.33, 125.17, 123.89, 121.72, 120.88, 118.67, 110.17, 108.62, 74.83, 73.47, 57.11, 56.57, 56.25, 56.00, 30.05, 29.49, 29.44, 29.33, 29.31, 29.23, 28.96, 27.00, 26.56, 25.71, 25.48; HRMS (ESI) calculated for $C_{32}H_{43}BrN_2O_7$ $[M\text{-}Br]^+$ 567.3065, found 567.3074.

4.1.3.6. Synthesis of the title compound 15

Compound **15** was prepared from compound **14** by the same method as compound **13.**

4.1.3.6.1. Compound 15a

Yellow solid, yield 48%. m.p. 188 – 190 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.47 (s, 1H), 8.15 (d, *J* = 9.3 Hz, 1H), 7.93 (d, *J* = 9.4 Hz, 1H), 7.85 (s, 1H), 6.86 (s, 1H), 5.28 (s, 2H), 4.51 (t, *J* = 6.7 Hz, 2H), 4.42 (t, *J* = 6.6 Hz, 2H), 4.06 (s, 3H), 3.97 (s, 3H), 3.93 (s, 3H), 3.27 (s, 2H), 2.08 – 1.94 (m, 2H), 1.77 – 1.62 (m, 2H), 1.51 (s, 2H), 1.36 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.82, 151.20, 147.22, 147.16, 145.46, 136.45, 133.07, 132.46, 126.27, 123.30, 121.54, 118.77, 118.74, 114.01, 110.34, 75.78, 73.62, 58.30, 57.02, 56.41, 56.33, 30.14, 29.15, 29.00, 28.09, 26.68, 25.55, 25.52; HRMS (ESI) calculated for $C_{28}H_{34}BrN_2O_7$ $[M\text{-}Br]^+$ 589.1552, found 589.1544.

4.1.3.6.2. Compound 15b

Yellow solid, yield 43%. m.p. 184 – 186 ^{o}C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.46 (s, 1H), 8.14 (d, *J* = 9.3 Hz, 1H), 7.93 (d, *J* = 9.4 Hz, 1H), 7.85 (s, 1 H), 6.86 (s, 1H), 5.27 (s, 2H), 4.53 (t, *J* = 6.6 Hz, 2H), 4.41 (t, *J* = 6.6 Hz, 2H), 4.06 (s, 3H), 3.97 (s, 3H), 3.93 (s, 3H), 3.27 (s, 2H), 2.06 – 1.93 (m, 2H), 1.77 – 1.60 (m, 2H), 1.49 (s, 2H), 1.29 (s, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.83, 151.20, 147.23, 147.18, 145.53, 136.43, 133.08, 132.42, 126.29, 123.26, 121.54, 118.75, 114.02, 110.34, 75.88, 73.57, 58.29, 57.01, 56.41, 56.33, 30.23, 29.45, 29.39, 29.33, 29.07, 28.08, 26.69, 25.68, 25.60; HRMS (ESI) calculated for $C_{30}H_{38}BrN_2O_7$ $[M\text{-}Br]^+$ 617.1856, found 617.1857.

4.1.3.6.3. Compound 15c

Yellow solid, yield 44%. m.p. 183 – 185 ^{o}C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.52 (s, 1H), 8.17 (d, *J* = 9.3 Hz, 1H), 7.95 (d, *J* = 9.4 Hz, 1H), 7.89 (s, 1H), 6.89 (s, 1H), 5.31 (s, 2H), 4.57 (t, *J* = 6.8 Hz, 2H), 4.45 (t, *J* = 6.7 Hz, 2H), 4.09 (s, 3H), 4.00 (s, 3H), 3.96 (s, 3H), 3.32 (t, *J* = 5.6 Hz, 2H), 2.11 – 1.97 (m, 2H), 1.79 – 1.66 (m, 2H), 1.59 – 1.46 (m, 2H), 1.45 – 1.24 (m, 14H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 151.83, 151.21, 147.31, 147.23, 145.65, 136.44, 133.10, 132.50, 126.21, 123.20, 121.59, 118.76, 118.70, 114.03, 110.34, 75.96, 73.56, 58.30, 56.99, 56.41, 56.32, 30.27, 29.63, 29.58, 29.48, 29.37, 29.10, 28.11, 26.71, 25.73, 25.61; HRMS (ESI) calculated for $C_{32}H_{42}BrN_2O_7$ $[M\text{-}Br]^+$ 645.2170, found 645.2171.

4.2. Pharmacology

4.2.1. *In vitro* antiproliferative assay

HepG2, SMMC-7721, HCT-116, HL-60, and LO-2 cells were purchased from Nanjing Key Gen Biotech Co. Ltd. (Nanjing, China). The cytotoxicity of the test compounds were determined using MTT assay. Briefly, the cell lines were incubated at 37 °C in a humidified 5% CO_2 incubator for 24 h in 96-microwell plates. After medium removal, 100 μL of culture medium with 0.1% DMSO containing the test compounds at different concentrations was added to each well and incubated at 37 °C for another 72 h. The MTT (5 mg/mL in PBS) was added and incubated for another 4 h, the optical density was detected with a microplate reader at 490 nm. The IC_{50} values were calculated according to the dose-dependent curves. All the experiments were repeated in at least three independent experiments.

4.2.2. Griess assay

The levels of NO produced by selected compounds were determined by reaction of nitrite ion with Griess reagent. Briefly, 100 μM of test compound in PBS containing 2% dimethyl sulfoxide and 5.0 mM *L*-cysteine at pH 7.4 were incubated at 37 °C in acellular or HepG2 cell lysates for the indicated time, and sampled at the specified time point. The collected samples were mixed with Griess reagent and incubated at 37 °C for 15 min, followed by measuring at 540 nm using a Microplate Reader (Tecan, Männedorf, Switzerland). Each compound was measured in triplicate. Standard sodium nitrite solutions at different concentrations were used to construct the calibration curve, from which the amount of nitric oxide release (quantitated as nitrite ion) was calculated.

4.2.3. Analysis of cell cycle arrest

HepG2 cells were seeded into 6-well plates and incubated at 37 ^{o}C in a humidified 5% CO2 incubator for 24 h, and then treated with or without **15a** at indicated concentrations for another 72 h. The collected cells were fixed by adding 70% ethanol at 4 ^{o}C for 12 h. Subsequently, the cells were resuspended in PBS containing 100 μL RNase A and 400 μL of propidium iodide for 30 min.

The DNA content of the cells was measured using a FACS Calibur flow cytometer (BectoneDickinson, San Jose, CA, USA).

4.2.4. Analysis of cellular apoptosis

After treatment with or without **15a** at indicated concentrations for 72 h, the cells were washed twice in PBS, centrifuged and resuspended in 500 μL AnnexinV binding buffer. The cells were then harvested, washed and stained with 5 μL Annexin V-APC and 5 μL 7-AAD in the darkness for 15 min. Apoptosis was analyzed using a FACS Calibur flow cytometer (BectoneDickinson, San Jose, CA, USA).

4.3.5. Detection of mitochondrial membrane potential

HepG2 cells were incubated with **15a** as described above. After incubation for 72 h, the cells were washed in PBS and resuspended in 500 μL JC-1 incubation buffer at 37 ^{o}C for 15 min. Then, **15a** was immediately assessed for a red fluorescence using a microplate reader (ELx80, Bio-Tek, USA). The fluorescent signal of monomers was measured with an excitation wavelength of 488 nm. The percentage of cells with healthy or collapsed mitochondrial membrane potentials was monitored by flow cytometry analysis (BectoneDickinson, San Jose, CA, USA).

4.2.6. *In vivo* antitumor assay

Five-week-old male Institute of Cancer Research (ICR) mice were purchased from Shanghai SLAC Laboratory Animals Co. Ltd. A total of 1×10^{6} H22 cells were subcutaneously inoculated into the right flank of ICR mice according to protocols of tumor transplant research, to initiate tumor growth. After incubation for one day, mice were weighted and at random divided into five groups of eight animals. The groups treated with palmatine, **15a** were administered 30, 15, 30 mg/kg in a vehicle of 10% DMF/2% Tween 80/88% saline, respectively. Cyclophosphamide (20 mg/kg) was used as positive control and vehicle as negative control by intravenous injection. Treatments were done at a frequency of intravenous injection one dose per day for a total 21 consecutive days. The mice were sacrificed after the treatments and the tumors were excised and weighed. The inhibition rate was calculated as follows: Tumor inhibitory ratio (%) = (1-average tumor weight of treated group/average tumor weight of control group) × 100%.

Acknowledgments

This study was financially supported by the National Natural Science Foundation of China (No. 81373280, 81673306), the Project Program of the State Key Laboratory of Natural Medicines, China Pharmaceutical University (No. SKLNMKF201710), and the Innovation project of Jiangsu Province (No. KYLX15_0633).

Appendix A. Supplementary data

Supplementary data related to this article can be found at

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1. Eighteen novel NO-donating protoberberine derivatives were synthesized.
2. Most compounds showed significantly enhanced *in vitro* anti-proliferative activity.
3. **15a** exhibited good selectivity between tumor cells and normal liver LO-2 cells.
4. The antitumor activity of **15a** in HepG2 cells was diminished by an NO scavenger.
5. **15a** effectively inhibited liver tumor growth in an *in vivo* mouse model.