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ARTICLE TYPE

Synthesis and biological evaluation of piperzaine group linked bivalent β-carbolines as potential antitumor agents

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A series of novel bivalent β-carbolines with a spacer of piperazine group between the 3-methylene was synthesized and evaluated as antitumor agents. The results demonstrated that the compounds **7e** and **7g** exhibited the most potent cytotoxic activities against ten tumor cell lines. Structure-activity relationships analysis indicated that (1) the substituent in position-1 and 9 of β-carboline ring play a significant role in modulation of antitumor activity; (2) the introduction of alkyl groups into position-9 of β-carboline nucleus facilitated their cytotoxic potencies and the butyl substituent was the optimal group. The preliminary mechanism of action investigation demonstrated that compound **7g** showed obvious anti-angiogenetic activity in the *in vivo* CAM assay, and the potency was similar to CA4P (200μM).

Introduction

The β-carboline alkaloids are a large group of natural and synthetic indole alkaloids with a tricyclic pyrido[3,4-b]indole ring system, and possess a broad spectrum of biochemical effects and pharmaceutical functions. [1] Recently, many researches have done much to design and synthesize many novel β-carbolines as a new class of antitumor agents.[2-9] These compounds were discovered to function their antitumor activities through multiple mechanisms, which include intercalating into DNA,[10] inhibiting Topo I and II (topoisomerase I and II), [11] CDK (cyclin-dependent kinase), [12] MK-2 (mitogen activated protein kinase-activated protein kinase 2), [13] kinesin-like protein Eg5, [14] IKK (I-Kappa-B kinase)[15] and PLK (polo-like kinase). [16]

Our group previously reported the synthesis of a large quantity of β-carbolines as a new class of antitumor agents and the evaluation of their antitumor activities in *vitro* and in *vivo*.[17-28] Structure-activity relationships analysis of these compounds indicated that (i) the common β-carboline moiety was very important for their potent antitumor activities; the introduction of appropriate substituents into position-1, 3 and 9 of β-carboline nucleus played a vital role in determining their antitumor potencies; (ii) the n-butyl and 3-phenylpropyl substituents in position 9 of β-carboline nucleus were the optimal pharmacophoric group giving rise to significant antitumor agents. Previous investigations[29] suggested that dimerization of various intercalating agents by an appropriate spacer could lead to a marked increase in the DNA binding affinity. Therefore, bivalent β-carbolines were expected to represent more potent antitumor efficacies than monomers. Recently, several bivalent β-carbolines were synthesized and evaluated as anti-Alzheimer's[30] and antitumor[31] agents (**Figure 1**) and bivalent β-carbolines were proved to exhibit more potent anti-Alzheimer potencies than monomers. However, to the best of our knowledge, no large, systematic study has been undertaken to examine the structure-activity relationships of bivalent β-carbolines as antitumor agents. We recently began such a study and have reported the synthesis *in vitro* evaluation, *in vivo* efficacies and structure-activity relationships for the new bivalent β-carbolines linked at the N-9 and C-3 position with a spacer of three to ten methylene units, respectively.[32-34] (**Figure 1**).

However, the bivalent β-carbolines mentioned above have limited utility for cancer therapy because of their poor solubility.

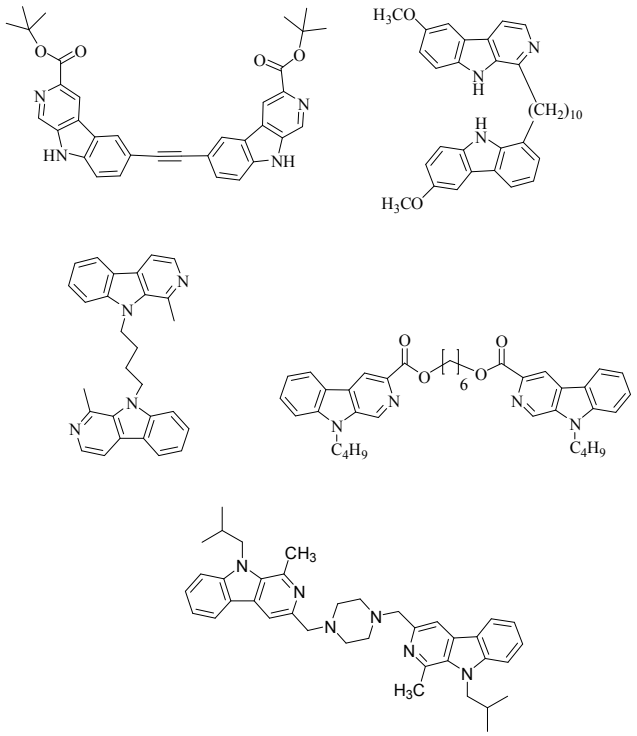
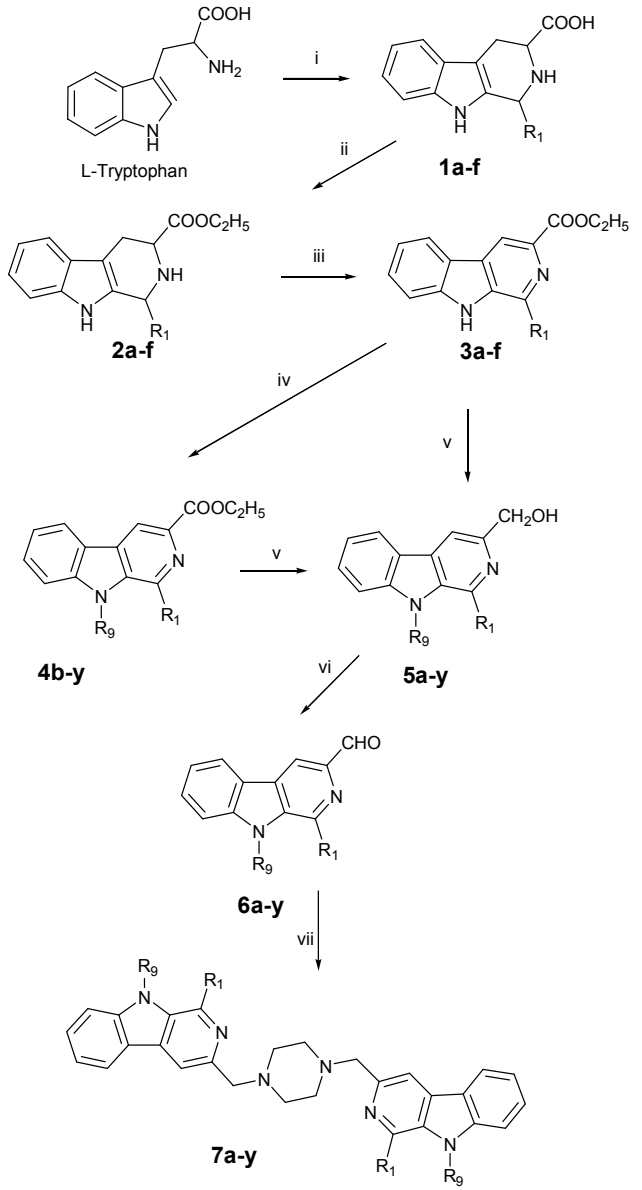


Figure 1 The chemical structure of the representative reported and newly synthesized bivalent β-carbolines

To circumvent the solubility problem and find congener more active as potential antitumor agents, in this investigation, we designed and synthesized a series of piperazine group linked bivalent β-carbolines as potent antitumor agents. These compounds were expected to exhibit significantly improved antitumor activities due to the improved water solubility. We report now the synthesis, *in vitro* evaluation, preliminary structure-activity relationships and mechanism of action for the new bivalent β-carbolines with a spacer of piperazine group between the 3-methylene.



Scheme 1 Synthesis of the bivalent β-carbolines **7a-y**.
Reagents and conditions: (**i**) acetic acid, R^1CHO, reflux, 3 h; (**ii**) ethanol, $SOCl_2$, reflux, 4 h; (**iii**) xylene, S, reflux, 8 h; (**iv**) DMF, NaH, alkyl halogenide, stirred at RT; (**v**) THF, $LiBH_4$, stirred at RT; (**vi**) CH_3CN, MnO_2, reflux, 2 h; (**vii**) sym-dichloroethane, $NaBH_3CN$, stirred at RT.

Chemistry

The synthetic route of the bivalent β-carbolines **7a-y** was outlined in **Scheme 1**. Monovalent β-carbolines **3a-c**, **3f**, **4b-i**, **4w-y**, **5a-h**, **5v-y**, **6a-h** and **6v-y** were synthesized according to previously published methods. [17-19,21-23] The intermediate schiff base were prepared by the condensation of compounds **6a-y** with piperazine in anhydrous sym-dichloroethane at 60℃, and followed by reduction with $NaBH_3CN$ in anhydrous sym-dichloroethane at room temperature to afford compounds **7a-y** in 21-56% yield. The chemical structures of all the newly synthesized compounds were characterized by MS, HRMS, ^1H NMR and ^{13}C NMR.

Experimental section

Cytotoxicity *in vitro*

Cytotoxicity assays *in vitro* were carried out using 96 microtitre plate cultures and MTT staining according to the procedures described by Cao et al. [23] Briefly, cells were grown in RPMI-1640 medium containing 10% (v/v) fetal calf serum and 100 μM penicillin and 100 μM streptomycin. Cultures were propagated at 37℃ in a humified atmosphere containing 5% CO_2. Cell lines were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. DMSO was used as the solution for drugs. Final concentration of DMSO in the growth medium was 2% (v/v) or lower, concentration without effect on cell replication. In all of these experiments, three replicate wells were used to determine each point.

In vivo CAM assay

Antiangiogenic activity of the selected compound **7g** was investigated *in vivo* using chicken chorioallantoic membrane (CAM) assay. Five day-old fertilized eggs were obtained from local hatchery. 5 mL of albumin was aspirated and the eggs were incubated horizontally to allow the CAM to detach from the shell. Compound **7g** was prepared in 1.2% agarose discs at concentration of 200, 100 and 50μM/disc, respectively. Combretastain A4 phosphate (CA4P) was used as reference drug. Discs containing the vehicle only (DMSO) were used as negative control. A small window opening was made in the shell, and the discs were directly applied onto the CAM. The square opening was covered with sterilized surgical tape and the embryos were incubated for 48 h at 37°C. The CAMs were photographed under a dissecting microscope and blood vessels in each CAM were counted. The results are presented as a mean percentage of inhibition to the control ± SD, (n = 3).

Result and discussion

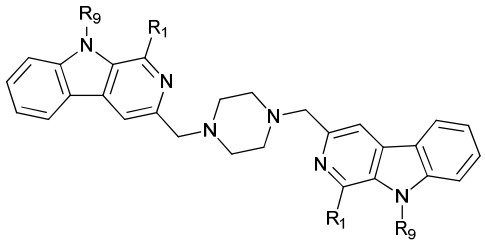
Cytotoxicity *in vitro*

The cytotoxic potencies of all the newly synthesized bivalent β-carbolines against a panel of human tumor cell lines and the LLC were investigated, and cisplatin was used as reference drug. In order to enhance the solubility in aqueous solution, all bivalent β-carbolines were prepared in the form of hydrochloride salt before use. As predicted, the hydrochloride salt of novel bivalent β-carbolines linked with a spacer of piperazine group in position-3 showed good water-solubility (more than 1.0 mg/ml). The results were summarized in **Table 1**.

As shown in **Table 1**, compounds **7b**, **7d**, **7h**, **7j**, **7o**, **7r**, **7s** and **7x** displayed significant and selective cytotoxicities with IC_{50} value lower than 20μM against some tumor cell lines. Compound **7e** showed the most potent cytotoxic activities with IC_{50} value of 7.62, 8.95, 5.32, 3.02, 8.35, 5.51, 7.62 and 5.15μM against MCF-7, HepG2, 22RV1, 769-P, A375, SK-OV-3, BGC-823 and LLC tumor cell lines. Similarly, compound **7g** had potent cytotoxic activities with IC_{50} value lower than 20μM against ten tumor cell lines. While compounds 7**a**, 7**c**, 7**f**, **7i-n**, **7p-q**, **7t-w** and **7y** exhibited weak to inactive cytotoxic activities.

We examined the influence of the substituents in position-9 of β-carboline ring on cytotoxic potencies. **Table 1** showed that compounds **7a, 7c** and **7d** having an ethyl, benzyl and 4-fluorobenzyl group in position-9 of β-carboline ring, respectively,

Table 1 Cytotoxic activity of bivalent β-carbolines *in vitro*



Compds	R_1	R_9	IC_{50} (μM) [a]									
			MCF-7 [b]	HepG2	22RV1	HT-29	769-P	A375	SK-OV-3	Eca-109	BGC-823	LLC
7a	H	C_2H_5	82.8	>100	52.3	32.8	64.3	49.9	24.4	>100	68.6	13.8
7b	H	n-C_4H_9	29.2	15.2	43.9	17.1	38.2	16.8	11.3	26.4	9.52	2.36
7c	H	$PhCH_2$	>100	65.4	>100	36.8	>100	>100	46.5	>100	>100	79.8
7d	H	(4-F)$PhCH_2$	72.8	76.3	60.6	18.3	29.0	46.0	8.43	24. 7	85.6	15.9
7e	H	$Ph(CH_2)_3$	7.62	8.95	5.32	18.7	3.02	8.35	5.51	24.6	7.62	5.15
7f	CH_3	CH_3	>100	>100	86.3	>100	>100	>100	31.0	>100	78.5	61.8
7g	CH_3	i-Bu	7.16	9.66	12.5	11.3	12.74	13.0	16.0	14.3	10.8	7.68
7h	CH_3	(3-Cl)$PhCH_2$	43.7	38.1	40.8	25.2	45.8	32.4	51.4	57.6	40.8	12.0
7i	Ph	CH_3	>100	>100	54.3	>100	>100	40.9	>100	>100	>100	34.5
7j	Ph	C_2H_5	>100	>100	24.92	17.6	29.6	9.92	19.8	>100	38.8	14.0
7k	Ph	$PhCH_2$	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
7l	Ph	$Ph(CH_2)_3$	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
7m	(4-OCH_3)Ph	H	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
7n	(4-OCH_3)Ph	CH_3	>100	55.0	47.8	29.0	>100	45.7	57.8	68.5	56.3	69.0
7o	(4-OCH_3)Ph	n-C_4H_9	20.6	25.7	27.6	11.8	16.6	19.6	24.6	24.7	28.8	27.4
7p	(4-OCH_3)Ph	$PhCH_2$	>100	>100	>100	>100	>100	>100	>100	>100	>100	40.4
7q	(4-OCH_3)Ph	$Ph(CH_2)_3$	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
7r	3,4-di(OCH_3)Ph	C_2H_5	27.3	27.2	42.3	34.3	61.1	37.3	32.7	68.5	19.8	9.68
7s	3,4-di(OCH_3)Ph	n-C_4H_9	14.5	17.0	19.5	11.8	10.0	15.5	25.7	32.4	6.21	9.78
7t	3,4-di(OCH_3)Ph	$PhCH_2$	>100	>100	>100	>100	>100	>100	>100	>100	>100	36.4
7u	3,4-di(OCH_3)Ph	$Ph(CH_2)_3$	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
7v	3,4,5-tri(OCH_3)-Ph	H	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
7w	3,4,5-tri(OCH_3)-Ph	CH_3	65.6	>100	>100	>100	>100	64.2	18.9	26.3	45.3	39.0
7x	3,4,5-tri(OCH_3)-Ph	n-C_4H_9	12.5	7.26	6.82	13.5	15.6	36.0	7.11	18.3	26.6	19.8
7y	3,4,5-tri(OCH_3)-Ph	$Ph(CH_2)_3$	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
	Cisplatin		3.70	46.6	3.70	>100	14.7	5.23	52.3	70.6	29.4	9.97

[a]Cytotoxicity as IC_{50} for each cell line, is the concentration of compound which reduced by 50% the optical density of treated cells with respect to untreated cells using the MTT assay. The data represents mean values±SD of at least three independent experiments. Values > 100 μM indicate less than 50% growth inhibition at > 100 μM.
[b]Cell lines include breast carcinoma (MCF-7), liver carcinoma (HepG2), prostate carcinoma (22RV1), colon carcinoma (HT-29), renal carcinoma (769-P), malignant melanoma (A375), ovarian carcinoma (SK-OV-3), esophageal carcinoma (Eca-109), gastric carcinoma (BGC-823) and Lewis lung carcinoma (LLC).

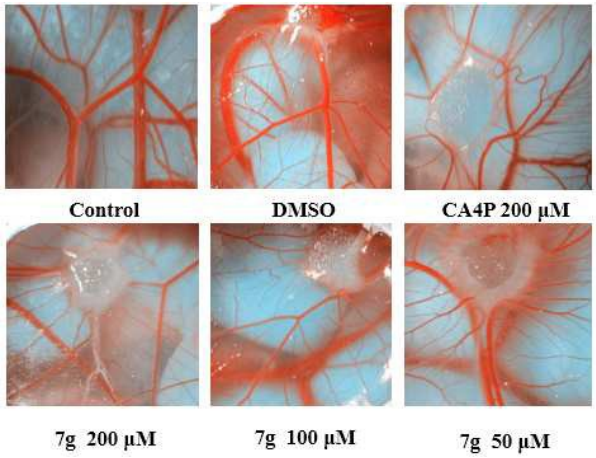


Figure 2 Inhibitory effects of compound **7g** on angiogenesis of CAM

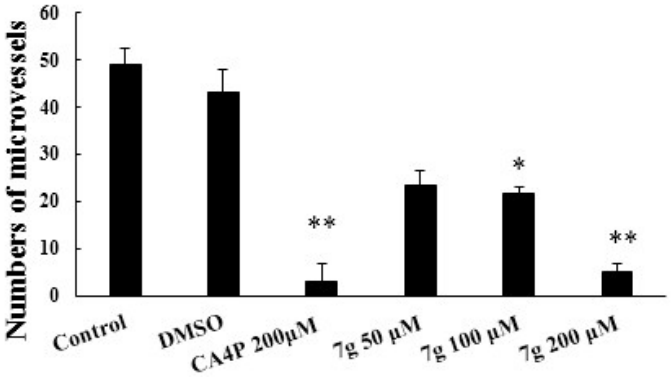


Figure 3 The anti-angiogenetic activity of compound **7g**

displayed weak to inactive cytotoxic activities, while compounds **7b** and **7e** bearing a butyl and 3-phenylpropyl group in position-9 of β-carboline ring, respectively, exhibited good to strong cytotoxic activities. Significantly, compound **7e** had the most potent cytotoxic activities with IC_{50} value lower than 10μM against eight tumor cell lines. Similarly, compounds **7g**, **7o**, **7s** and **7x** having a butyl group in position-9 of β-carboline ring, respectively, also exhibited good cytotoxic activities against all investigated tumor cell lines, while other compounds had weaker cytotoxic activities. These results suggested that the butyl substituent in position-9 of β-carboline ring were the most suitable group giving rise to potent cytotoxic agents.

Next, we examined the influence of the substituents in position-1 of β-carboline ring on cytotoxic potencies. The data collected in **Table 1** showed that compounds **7e** displayed significant and selective cytotoxic effects with IC_{50} value of lower than 20μM against most tumor cell lines. Introduction of a phenyl, 4-methoxyphenyl, 3,4-dimethoxyphenyl and 3,4,5-trimethoxyphenyl group into position-1 of compound **7e**, respectively, led to compound **7l**, **7q**, **7u** and **7y**, which were almost inactive against all tumor cell lines at the concentration of 100μM. In comparison with compounds **7b**, compounds **7o**, **7s** and **7x** bearing an additional 4-methoxyphenyl, 3,4-dimethoxyphenyl and 3,4,5-trimethoxyphenl group in position-1 of β-carboline ring, respectively, exhibited the similar cytotoxic activities against ten tumor cell lines. In addition, compounds 7l and **7k** having a 3-phenylpropyl and benzyl group in position-9 of β-carboline ring exhibited weaker cytotoxic potencies than compounds **7i** and **7j** which bearing a methyl and n-butyl group, respectively. Similarly, compounds **7m**, **7p**, **7q**, **7t**, **7u**, **7v** and **7y** with a hydrogen atom or arylated alkyl group in position-9 of β-carboline ring showed weaker cytotoxic activities, while other compounds had good and selective cytotoxic effects against tumor cell lines. These results indicated that the introduction aryl substituent into position-1 of β-carboline ring might be detrimental to cytotoxic effects of this class of compounds.

Anti-angiogenetic activitiy *in vivo*

The most active compound **7g** was selected to evaluate the angiogenetic activity by CAM assay. The inhibitory effects of compounds **7g** on angiogenesis of CAM are shown in **Figure 2**. The anti-angiogenetic activities of compounds **7g** was semiquantitatively analyzed using Graph Pad Prism 5.0. (shown in **Figure 3**). The result showed that compound **7g** ($p < 0.05$) could inhibit the angiogenesis of CAM. The anti-angiogenetic activity of compound **7g** was comparable with CA4P *in vivo* CAM assay at the same dose (200μM).

Conclusions

In conclusion, we have synthesized a series of novel bivalent β-carbolines with a spacer of piperazine group between the 3-methylene, and investigated their cytotoxic potential against ten tumor cell lines in culture. Some compounds exhibited good and selective cytotoxic activities against ten tumor cell lines. The compounds **7e** and **7g** exhibited the most potent cytotoxic activities against ten tumor cell lines. Preliminary structure-activity relationships analysis indicated that the substituent in position-1 and 9 of β-carboline ring played a significant role in modulation of antitumor activity. The introduction of alkyl groups into position-9 of β-carboline nucleus provided compounds with greatly enhanced cytotoxic potencies and the butyl substituent in position-9 of β-carboline nucleus was the optimal group, while the introduction of arylated alkyl groups might be harmful to antitumor activities. Moreover, the most active compound **7g** was found to show obvious angiogenesis inhibitory effects in CAM assay, and the anti-angiogenetic potency was comparable to the reference drug CA4P. Further investigations to confirm antitumor efficacy in animal models and elucidate the pharmacological mechanisms of this class of compounds are underway in our laboratory, and the data will be published elsewhere.

Acknowledgments

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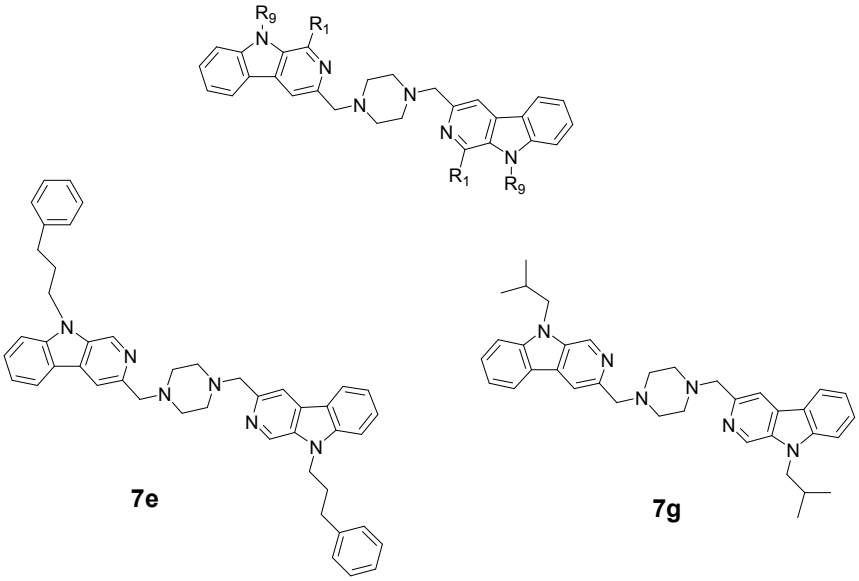
†Electronic Supplementary Information (ESI) available.

Graphic abstract



A series of bivalent β-carbolines with a spacer of piperazine group between the 3-methylene was synthesized and their cytotoxic activities *in vitro* were evaluated. Compounds **7e** and **7g** exhibited potent cytotoxic activity against ten tumor cell lines.