Bioorganic Chemistry 96 (2020) 103612

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Molecular hybrid design, synthesis, *in vitro* and *in vivo* anticancer evaluation, and mechanism of action of *N*-acylhydrazone linked, heterobivalent β-carbolines

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ARTICLE INFO

Keywords:
Heterobivalent β-carboline
Acylhydrazone group
Cytotoxic activity
Structure-activity relationships

ABSTRACT

A series of *N*-acylhydrazone-linked, heterobivalent β-carboline derivatives was designed and synthesized from L-tryptophan in a nine-step reaction sequence. The effort resulted in the heterobivalent β-carbolines **10a–t** in good yields. The target compounds were characterized by ^{1}H NMR, ^{13}C NMR and high-resolution mass spectrometry (HRMS). The *in vitro* cytotoxic activity of the synthesized compounds was evaluated against normal EA.HY926 cells and five cancer cell lines: LLC (Lewis lung carcinoma), BGC-823 (gastric carcinoma), CT-26 (murine colon carcinoma), Bel-7402 (liver carcinoma), and MCF-7 (breast carcinoma). Compound **10e**, with an IC_{50} value of 2.41 μM against EA.HY926 cells, was the most potent inhibitor. It showed cytotoxicity against all five cancer cell lines of different origin – murine and human, with IC_{50} values ranging from 4.2 ± 0.7 to 18.5 ± 3.1 μM. A study of structure-activity relationships indicated that the influence on cytotoxic activities of the substituent in the R_9′-position followed the tendency, 2,3,4,5,6-perfluorophenylmethyl > 4-fluorobenzyl > 3-phenylpropyl group. The antitumor efficacies of the selected compounds were also evaluated in mice. Compound **10e** exhibited potent antitumor activity, with tumor inhibition of more than 40% for Sarcoma 180 and 36.7% for Lewis lung cancer. Furthermore, the pharmacological mechanisms showed that compound **10e** has a certain impairment in the motility of LLC cells, which suggests the anti-metastatic potential. And compound **10e** inhibited angiogenesis in chicken chorioallantoic membrane assay, and the anti-angiogenetic potency was more potent than the reference drug combretastatin A4-phosphate (CA4P) at a concentration 50 μM.

1. Introduction

Cancer is a serious clinical problem affecting millions of patients [1]. The occurrence of cancer is increasing due to the increase and aging of the world population. The World Health Organization has estimated that, by 2030, there will be 15 million deaths due to cancer worldwide [2]. Although extensive efforts have been made to deal with this situation, the methods for management of cancer remain limited. In pharmaceutical research, the search for new, effective, and safe chemotherapeutic agents for treatment of this disease remains a challenge.

β-Carboline alkaloids are a class of natural and synthetic products that have a broad spectrum of biochemical effects and pharmacological properties, including nervous system [3], antimicrobial [4], and antineoplasmic treatments [5]. They are also effective in the treatment of dermatoses [6]. In recent decades, there have been intense research efforts in the design and development of β-carbolines as a new class of antitumor agents, and a large number of β-carboline derivatives have been prepared in search of agents that are more potent [7–11]. The results indicate that this class of compounds exert antitumor effects through various mechanisms of action, including intercalating with DNA [12,13], and by inhibition of cyclin-dependent kinase (CDK) [14], topoisomerases I and II [7,12,15], polo-like kinase 1 (PLK1) [16,17], and IκB kinase [18].

The bioactive acylhydrazone (−CONHN=) scaffold has been widely applied in medicinal chemistry, and it is often used in the design of drugs with heterocyclic moieties. Relative to amide groups, it endows molecules with better thermal stability as well as with hydrolytic and chemical stability [19,20]. The acylhydrazone group is present in numerous compounds that act on various types of molecular targets [21–23]. Among the pharmacological effects of acylhydrazone-derived compounds are anti-inflammatory and anticancer activities [24,25]. For example, aldoxorubicin (Fig. 1) is currently in a phase III trial for the

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https://doi.org/10.1016/j.bioorg.2020.103612
Received 6 June 2019; Received in revised form 25 December 2019; Accepted 20 January 2020
Available online 23 January 2020
0045-2068/

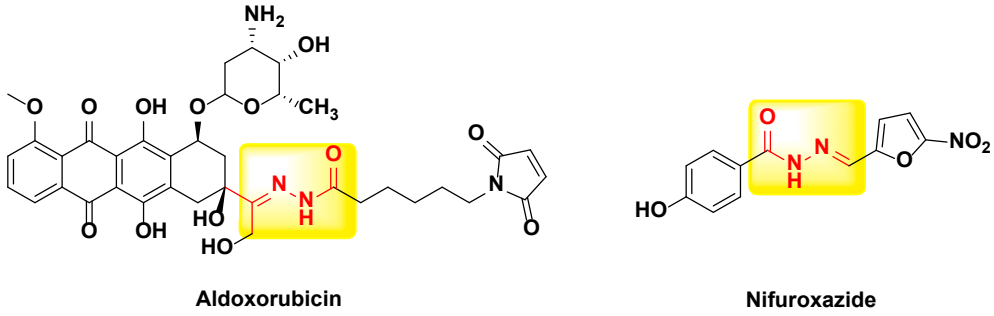


Fig. 1. Structures of *N*-acylhydrazone derivatives Aldoxorubicin and Nifuroxazide.

treatment of metastatic, locally advanced, or unresectable soft tissue sarcoma [26]. This (maleinimidoalkanoyl)hydrazone derivative of doxorubicin covalently binds to serum albumin through its maleimide moiety. Nifuroxazide (Fig. 1), another *N*-acylhydrazone derivative, is a nitrofuran antibiotic commonly used as an intestinal anti-infective agent [27].

Our research group [28–31] has focused on incorporating substituents into positions 1, 2, 3, 7, and 9 of the β-carboline nucleus as antitumor agents. Structure-activity relationship (SAR) analysis indicated that (1) the β-carboline moiety was associated with their potential antitumor activities; and (2) the introduction of appropriate substituents into positions 1, 3, and 9 of the β-carboline nucleus enhanced their antitumor potencies. Recently, we reported the SAR for the homobivalent β-carbolines and heterobivalent β-carbolines with an alkyl or alkylamino spacer in positions 1, 3, 7, and 9 of the β-carboline nucleus [32–37] (Fig. 2). Of these synthesized bivalent compounds, 1-Methyl-9-[4-(1-methyl-β-carboline-9-yl)butyl]-β-carboline (B-9-3) [36,38], which was a symmetric dimeric β-carboline compound that contains two molecules of harman bound to each other by a tetramethylene group, exhibited potent antitumor activity. Compounds B-1 [32], B-2 [37], and B-3 [33] exhibited significant angiogenesis inhibitory effects in CAM assay, and the anti-angiogenetic potency was comparable or more potent with the drug Endostar. The pharmacological mechanisms showed that B-9-3 selectively induces apoptosis of endothelial cells, in part through disruption of VEGF-A/VEGFR2 signaling [39], and also acts on the TGF-β signaling pathway [40].

Since molecular hybridization has been an effective approach for the development of new drugs, we utilized the tactic of combining the acylhydrazone and β-carboline moieties to develop a library of heterobivalent β-carboline derivatives, and to test their antitumor activities against cultured cells. In the present study, we reported synthesis, *in vitro* evaluation, *in vivo* efficacies and preliminary structure-activity relationships for the new *N*-acylhydrazone-linked, heterobivalent β-carboline derivatives.

2. Results and discussion

2.1. Chemistry

The target heterobivalent β-carboline derivatives **10a–t** were synthesized according to the procedures depicted in Schemes 1 and 2. The 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**2a**) and 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**2b**) were prepared by the condensation of L-tryptophan with appropriate aldehydes via the Pictet-Spengler condensation. Esterification of the intermediates **2a** with ethanol in the presence of thionylchloride ($SOCl_2$) allowed preparation of ethyl 1,2,3,4-tetrahydro-β-carboline-3-carboxylates **3a–b**; followed by dehydrogenation with sulfur in refluxing xylene, the monovalent β-carbolines **4a–b** were obtained. The N^9 of **4a–b** was alkylated by the action of sodium hydride (NaH) in anhydrous *N*,*N*-dimethylformamide (DMF), followed by the addition of 1-iodobutane or benzyl bromide to obtain compounds **5a–d**. The key intermediates **6a–d** were synthesized by the reaction of compounds **5a–d** with hydrazine monohydrate.

Subsequently, we focused on synthesis of intermediates **9a–e**. 1-Methyl-β-carboline **7** was obtained by the oxidation and decarboxylation of **2b** in a single step through the action of active manganese dioxide (MnO_2). From compound **7**, compounds **8a–e** were afforded according to a synthetic procedure similar to that used for compounds **5a–d**. The methyl group in position 1 of compounds **8a–e** was oxidized

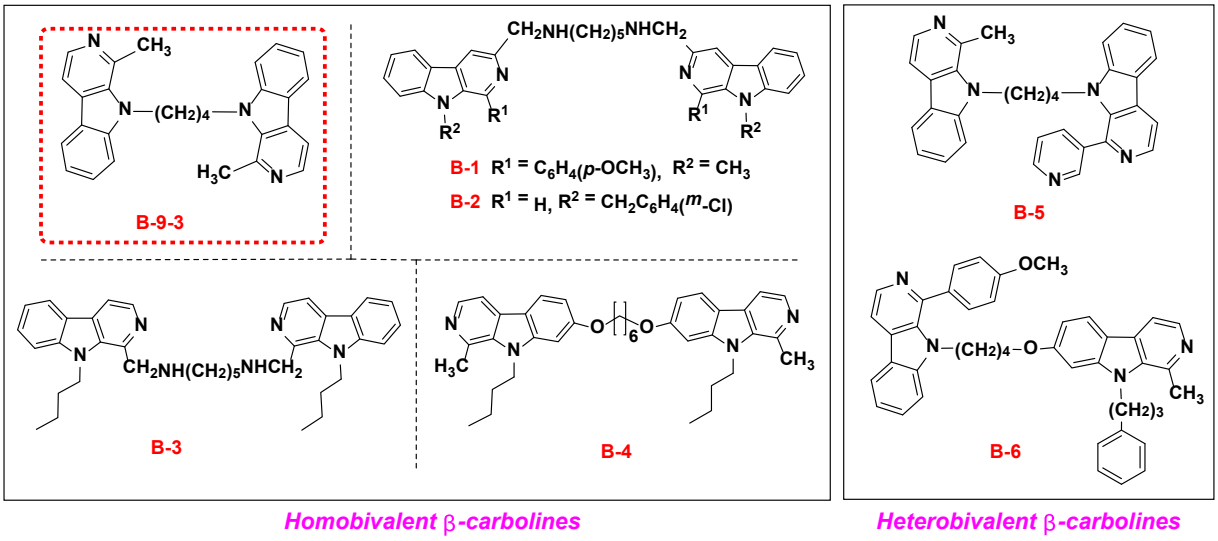
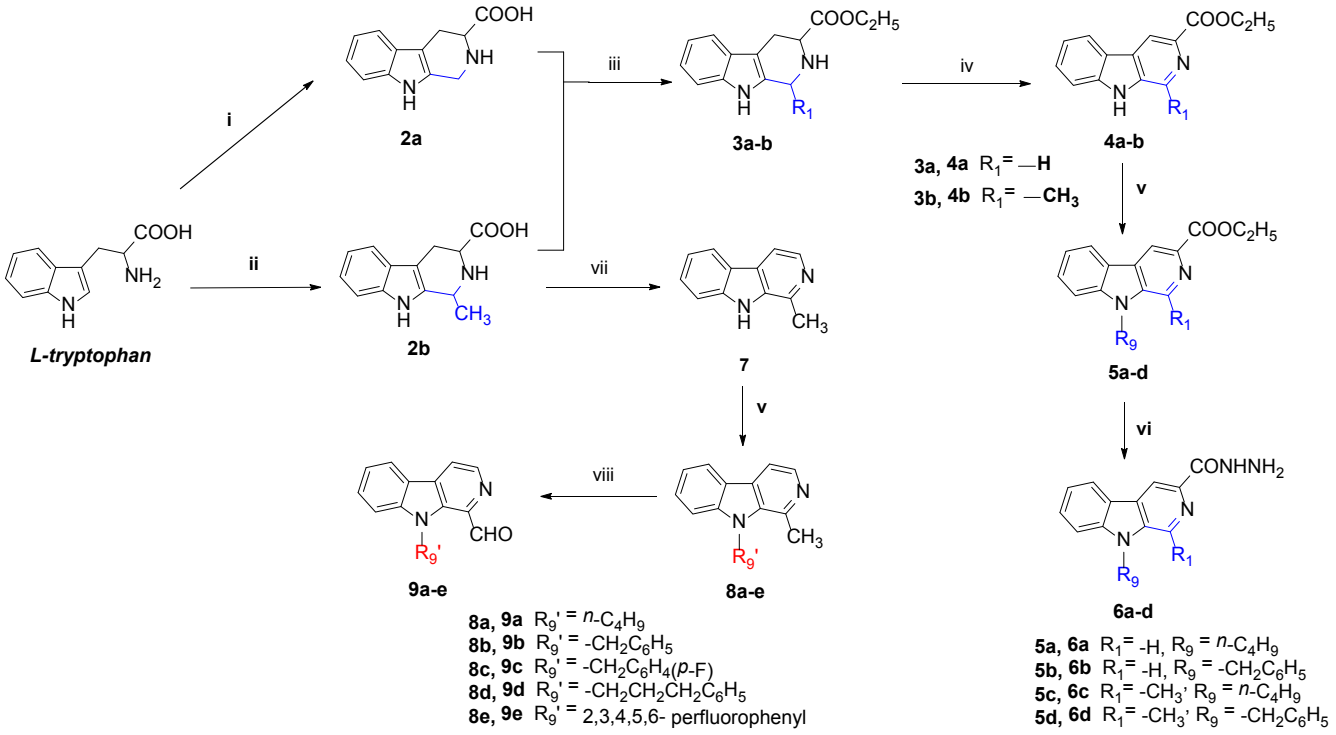


Fig. 2. The chemical structure of the representative reported bivalent β-carbolines.



Scheme 1. Synthesis of the key intermediate **6a-d, 9a-e**. Reagents and conditions: (i) NaOH, H_2O, formaldehyde, reflux, 3 h; (ii) H_2SO_4, H_2O, acetaldehyde, room temperature, 3 h; (iii) ethanol, $SOCl_2$, reflux, 4 h; (iv) xylene, S_8, reflux, 8 h; (v) DMF, NaH, alkyl halogenide, stirred at RT; (vi) hydrazine hydrate, ethanol, reflux, 4 h. (vii) H_2SO_4, MnO_2; (viii) SeO_2, dioxane, reflux, 2 h.

by selenium dioxide (SeO_2) in anhydrous dioxane to provide the β-carboline-1-carboxaldehydes **9a–e**.

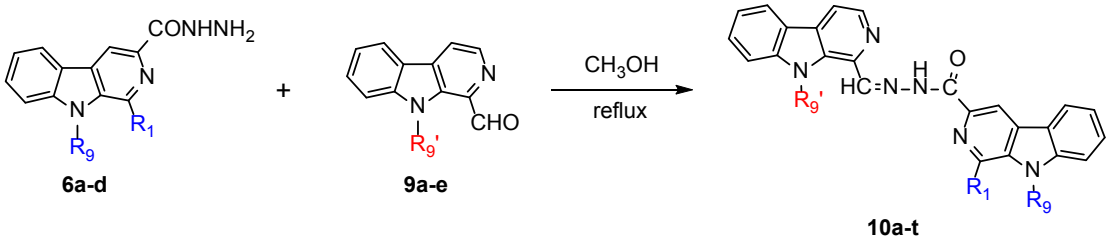
Finally, the title compounds **10a–t** were prepared in good yields from β-carboline-3-carbohydrazides **6a–d** with β-carboline-1-carboxaldehydes **9a–e**; the reactions were accomplished in ethanol under reflux conditions. The chemical structures of the synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR, and high-resolution mass spectrometry (HRMS).

2.2. Biological evaluation

2.2.1. Inhibitory effect on EA.HY926s proliferation

The 20 synthesized heterobivalent β-carbolines were evaluated against EA.HY926 cells for anti-proliferative effects by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), with CA4P and Endostar (rh-endostatin) as reference drugs.

As shown in Table 1, half of heterobivalent β-carbolines exhibited good anti-proliferative effects, with the half maximal inhibitory concentration (IC_{50}) values of less than 10.0 μM against EA.HY926 cells. Compounds **10c**, **10e**, **10n**, and **10o** displayed anti-proliferative



10a R_9' = R_9 = *n*-butyl, R_1 = H
10b R_9' = benzyl, R_1 = H, R_9 = *n*-butyl
10c R_9' = 4-fluorobenzyl, R_1 = H, R_9 = *n*-butyl
10d R_9' = 3-phenylpropyl, R_1 = H, R_9 = *n*-butyl
10e R_9' = 2,3,4,5,6- perfluorophenyl, R_1 = H, R_9 = *n*-butyl
10f R_9' = *n*-butyl, R_1 = H, R_9 = benzyl
10g R_9' = benzyl, R_1 = H, R_9 = benzyl
10h R_9' = 4-fluorobenzyl, R_1 = H, R_9 = benzyl
10i R_9' = 3-phenylpropyl, R_1 = H, R_9 = benzyl
10j R_9' = 2,3,4,5,6- perfluorophenyl, R_1 = H, R_9 = benzyl
10k R_9' = R_9 = *n*-butyl, R_1 = CH_3
10l R_9' = benzyl, R_1 = CH_3, R_9 = *n*-butyl
10m R_9' = 4-fluorobenzyl, R_1 = CH_3, R_9 = *n*-butyl
10n R_9' = 3-phenylpropyl, R_1 = CH_3, R_9 = *n*-butyl
10o R_9' = 2,3,4,5,6- perfluorophenyl, R_1 = CH_3, R_9 = *n*-butyl
10p R_9' = *n*-butyl, R_1 = CH_3, R_9 = benzyl
10q R_9' = benzyl, R_1 = CH_3, R_9 = benzyl
10r R_9' = 4-fluorobenzyl, R_1 = CH_3, R_9 = benzyl
10s R_9' = 3-phenylpropyl, R_1 = CH_3, R_9 = benzyl
10t R_9' = 2,3,4,5,6- perfluorophenyl, R_1 = CH_3, R_9 = benzyl

Scheme 2. Synthetic route for the heterobivalent β-carbolines (**10a-t**) preparation.

Table 1
Inhibitory effect of novel heterobivalent β-carbolines on EA.HY926 cell proliferation.

Compd.	IC_{50} (μM) ± SD	Compd.	IC_{50} (μM) ± SD
CA_4P	7.6 ± 1.3	**10j**	6.2 ± 0.8
Endostar	1.6 ± 0.5	**10k**	16.2 ± 2.9
10a	14.6 ± 3.3	**10l**	24.3 ± 4.6
10b	16.8 ± 2.7	**10m**	11.2 ± 2.3
10c	4.7 ± 0.6	**10n**	4.5 ± 0.7
10d	9.3 ± 2.3	**10o**	3.2 ± 0.4
10e	2.4 ± 0.8	**10p**	10.5 ± 1.6
10f	16.2 ± 3.2	**10q**	17.2 ± 3.2
10g	10.6 ± 2.6	**10r**	5.7 ± 0.7
10h	8.3 ± 2.8	**10s**	7.9 ± 0.9
10i	11.7 ± 3.17	**10t**	5.4 ± 1.2

potencies, with IC_{50} values of 4.7 ± 0.6, 2.4 ± 0.8, 4.5 ± 0.7, and 3.2 ± 0.4 against these cells, respectively. Compounds **10a–b**, **10d**, **10f–k**, **10m**, and **10p–t** showed only moderate anti-proliferative potencies, with IC_{50} values ranging from 5.4 ± 1.2 to 17.2 ± 3.2 μM. Unfortunately, compound **10l**, with an IC_{50} value of 24.3 ± 4.6 μM, had marginal anti-proliferative effects. However, compound **10e**, with an IC_{50} value of 2.4 ± 0.8 μM against EA.HY926 cells, was the most potent inhibitory agent; its potency was greater than that for the positive control drug, CA4P (7.6 ± 1.3 μM), but less than that of another positive drug control, Endostar (1.6 ± 0.5 μM).

Of heterobivalent β-carboline derivatives **10a-j**, all bearing a *n*-butyl or benzyl substituent in R_9-position of β-carboline nucleus, while in another β-carboline core, the substituted group of the R_9'-position were *n*-butyl (**10a**, **10f**), benzyl (**10b**, **10g**), 4-fluorobenzyl (**10c**, **10h**), 3-phenylpropyl (**10d**, **10i**), and 2,3,4,5,6-perfluorophenylmethyl (**10e**, **10j**), respectively. The influence of substituent in R_9'-position on anti-proliferative effects followed the tendency of 2,3,4,5,6-perfluorophenylmethyl > 4-fluorobenzyl > 3-phenylpropyl group. Similarly, the anti-proliferative potencies of compounds **10p-t** with a same substituent group followed the same tendency.

We then found the following anti-proliferative effects trend based on the R_9'-position of the β-carboline nucleus: 2,3,4,5,6-perfluorophenylmethyl > 4-fluorobenzyl > 3-phenylpropyl group. There is no obvious difference between the R_1-position substituent group, which indicates that this position is not tolerated for modification.

2.2.2. In vitro cytotoxic screening

The cytotoxic potencies of the heterobivalent β-carbolines against a panel of tumor cell lines were determined and compared with that of a reference drug, cisplatin. The tumor cell panel included LLC (Lewis lung carcinoma), BGC-823 (gastric carcinoma), CT-26 (murine colon carcinoma), Bel-7402 (liver carcinoma) and MCF-7 (breast carcinoma) of different origin – murine and human. The results are summarized in Table 2.

The cytotoxic activity results revealed that most of the newly designed compounds were active against the tested cell lines. Compounds **10b–e**, **10h–j**, **10m**, **10o**, and **10r–t** displayed a broad spectrum of cytotoxic activities, with IC_{50} values of less than 20 μM against four or five tumor cell lines; compounds **10a**, **10f–g**, **10l**, and **10q** showed cytotoxicities with IC_{50} values of less than 20 μM against one or two tumor cell lines.

Introduction of methyl groups on the R_1-position of the β-carboline ring of compounds **10a–j** yielded the compounds **10k–t**. A comparison of the cytotoxicities of compounds **10a–j** and **10k–t** revealed that omission of the methyl group in the R_1-position did not have a strong influence on cytotoxicity (**10a** vs. **10k**, **10e** vs. **10o**, **10h** vs. **10r**, and **10i** vs. **10s**). Introduction of a *n*-butyl substituent into the R_9-position of the β-carboline nucleus enhanced antitumor activities, as seen for compound **10b**, for which the antitumor activity was better than that of compound **10g** (except for the BGC823 cell line), and the activity of compound **10e** was superior to that of compound **10j** (except for the MCF-7 cell line). Compounds **10d**, **10k**, **10l**, and **10o**, with *n*-butyl groups in the R_9-position of the β-carboline nucleus, showed the same tendency, suggesting that the R_9 substituent affected the cytotoxic activities and that a *n*-butyl substitution was more favorable than a benzyl group.

The effect of various substituents on the R_9'-position of the β-carboline core was examined, and SAR studies revealed that the introduction of aromatic substituents was necessary for potent cytotoxicity. Compounds **10a–e** contained *n*-butyl in R_9-position of the β-carboline core, and, in addition, *n*-butyl (**10a**), benzyl (**10b**), 4-fluorobenzyl (**10c**), 3-phenylpropyl (**10d**), or 2,3,4,5,6-perfluorophenylmethyl (**10e**) in the R_9'-position of another β-carboline ring. Of these five heterobivalent β-carbolines, compound **10e** displayed higher cytotoxic activities against LLC, BGC-823, CT-26, and Bel-7402 cells, with IC_{50} values of 7.6 ± 0.8, 7.7 ± 1.2, 8.4 ± 1.1, and 4.2 ± 0.7 μM, respectively. Additionally, compounds **10f–j**, bearing benzyl in the R_9-position of the β-carboline core, and additional *n*-butyl, benzyl, 4-fluorobenzyl, 3-phenylpropyl, or 2,3,4,5,6-perfluorophenylmethyl groups in the R_9'-position of another β-carboline ring, and compound **10j**, showed IC_{50} values ranging from 9.2 ± 0.4 to 15.3 ± 0.9 μM.

Compared to cisplatin, some of these compounds displayed greater cytotoxicity against these tumor cell lines. In the cytotoxicity assay with the LLC cell line, compounds **10b–e**, **10h–j**, **10m–p**, and **10r–t** exhibited greater cytotoxicity than cisplatin (IC_{50} = 21.3 ± 1.1 μM), with IC_{50} values in the range of 6.7 ± 0.4 to 18.6 ± 2.7 μM. In the cytotoxicity assay with Bel-7402 cells, compounds **10c**, **10e**, **10h–j**, **10m**, **10o**, and **10q–t** showed higher cytotoxicity than cisplatin (IC_{50} = 15.4 ± 1.9 μM), with IC_{50} values in the range of 4.2 ± 1.9 to 13.3 ± 1.2 μM. In contrast, the IC_{50} value for compound **10e** against the BGC823 cell line was 7.7 ± 1.2 μM, as compared to that of cisplatin (IC_{50} = 8.4 ± 0.7 μM), but most compounds exhibited activities against this cell line, with IC_{50} values lower than 20 μM (except for **10a**, **10l**, and **10q**). Similarly, the IC_{50} value of compound **10j** against the MCF-7 cell line was 9.5 ± 1.1 μM, as compared to that of cisplatin, IC_{50} = 10.5 ± 2.3 μM. Compounds **10e** and **10j** both bear 2,3,4,5,6-perfluorophenylmethyl in the R_9'-position of the β-carboline ring.

2.3. Acute toxicity studies with healthy mice and in vivo antitumor activity

Since compounds **10e**, **10j**, **10o**, and **10t** showed the most cytotoxic activity, the antitumor activities of these compounds were evaluated against mice bearing Sarcoma 180 or Lewis lung cancer, and the results were compared with that for cyclophosphamide (CTX).

The LD_{50} (median lethal dose) values of the selected compounds after intraperitoneal (i.p.) administration to mice are listed in Table 3. Of these four investigated heterobivalent β-carboline derivatives, all had a 2,3,4,5,6-perfluorophenylmethyl group in the R_9-position of the β-carboline ring, and they displayed acute toxicity with LD_{50} values in the range of 50–85 mg kg^{-1}.

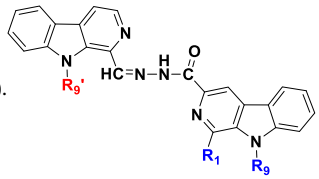
Our previous investigation [34] demonstrated that, in mice, Lewis lung cancer cells were more susceptible to β-carbolines than other animal models; therefore, this animal model was selected and evaluated. The tumor inhibition rates of all of the compounds are presented in Table 3. The highest tumor inhibition was 46.8% for the group with Sarcoma 180 and treated with compound **10e**; the tumor inhibitions of compounds **10j**, **10o**, and **10t** were 37.9%, 33.5%, and 39.2%, respectively. The tested compounds showed moderate antitumor activities for Lewis lung cancers with tumor inhibitions ranging from 21.2 to 36.7% at doses ranging from 10 to 17 mg/kg.

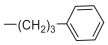
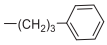
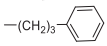
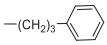
2.4. Inhibitory effect of 10e on tumor cell migration

In the present study, to explore whether compound **10e** could

Table 2

Cytotoxic activities of derivatives *in vitro* (IC_{50}, μM).



Compd.	R_9'	R_1	R_9	IC_{50} (μM) ± SD [a]				
				LLC[b]	BGC823	CT-26	Bel-7402	MCF-7
10a	*n*-butyl	H	*n*-butyl	39.3 ± 4.2	32.7 ± 3.7	15.1 ± 2.5	44.2 ± 3.3	24.5 ± 4.6
10b		H	*n*-butyl	17.7 ± 3.1	18.8 ± 2.7	13.3 ± 1.3	30.1 ± 4.6	19.1 ± 3.2
10c		H	*n*-butyl	14.0 ± 2.4	9.3 ± 0.8	19.6 ± 2.1	12.9 ± 1.4	32.9 ± 1.9
10d		H	*n*-butyl	16.3 ± 2.2	9.5 ± 0.6	17.1 ± 2.5	32.3 ± 2.7	16.5 ± 3.1
10e		H	*n*-butyl	7.6 ± 0.8	7.7 ± 1.2	8.4 ± 1.1	4.2 ± 0.7	18.5 ± 3.1
10f	*n*-butyl	H		28.1 ± 2.9	19.6 ± 2.7	19.8 ± 1.3	28.2 ± 4.1	96.1 ± 13.2
10g		H		29.6 ± 3.8	11.7 ± 2.2	19.2 ± 3.4	33.4 ± 6.4	34.3 ± 3.9
10h		H		6.7 ± 0.4	12.3 ± 0.7	21.7 ± 3.3	9.9 ± 1.7	12.8 ± 2.5
10i		H		8.7 ± 0.9	11.7 ± 3.2	19.6 ± 5.2	14.1 ± 4.3	20.6 ± 2.7
10j		H		9.2 ± 0.4	15.3 ± 0.9	10.5 ± 1.2	11.6 ± 2.3	9.5 ± 1.1
10k	*n*-butyl	CH_3	*n*-butyl	33.8 ± 5.2	18.6 ± 3.6	24.5 ± 2.3	18.8 ± 3.1	16.5 ± 3.4
10l		CH_3	*n*-butyl	56.7 ± 8.2	41.4 ± 5.1	14.9 ± 2.1	> 100	40.3 ± 3.3
10m		CH_3	*n*-butyl	8.4 ± 1.2	10.1 ± 1.7	7.6 ± 0.8	13.3 ± 1.2	17.2 ± 3.4
10n		CH_3	*n*-butyl	12.2 ± 1.7	14.1 ± 0.8	61.7 ± 5.1	62.6 ± 10.9	17.9 ± 3.8
10o		CH_3	*n*-butyl	7.8 ± 0.7	11.2 ± 0.4	9.8 ± 1.1	4.7 ± 0.7	12.5 ± 2.3
10p	*n*-butyl	CH_3		18.6 ± 2.7	19.5 ± 4.2	> 100	74.6 ± 9.7	19.3 ± 4.4
10q		CH_3		> 100	24.4 ± 4.1	18.6 ± 2.5	8.3 ± 0.7	> 100
10r		CH_3		6.9 ± 0.9	14.6 ± 0.7	13.6 ± 1.2	9.5 ± 1.7	17.2 ± 2.4
10s		CH_3		11.7 ± 1.6	9.3 ± 0.6	12.2 ± 0.7	11.5 ± 2.2	32.0 ± 4.7
10t		CH_3		7.4 ± 0.5	8.7 ± 1.4	13.5 ± 2.3	7.2 ± 0.6	16.2 ± 1.4
Cisplatin				21.3 ± 1.1	8.4 ± 0.7	4.2 ± 0.7	15.4 ± 1.9	10.5 ± 2.3

[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 represent the mean values ± SD of at least three independent determinations. Values > 100 μM indicate less than 50% growth inhibition at > 100 μM.

[b] Cell lines include Lewis lung carcinoma (LLC), gastric carcinoma (BGC), murine colon carcinoma (CT-26), liver carcinoma (Bel-7402), and breast carcinoma (MCF-7).

inhibit HT-29 and LLC cells migration, we performed cell migration assays. Cell migration plays an important role in tumor formation and cancer metastasis. It is relevant for angiogenesis to ensure tumor nutrition as well as for the formation of metastases, in which tumor cells leave the primary tumor site and spread to other tissues. The two cancer cell lines were treated with different concentrations of positive control CA4P and **10e** after 24 h were shown in Fig. 3. Compound **10e** inhibited cell migration of the two cancer cell lines dose-dependently.

2.5. Inhibition of angiogenesis in the chicken chorioallantoic membrane assay

A model system that has been used extensively to evaluate anti-angiogenic activity is the highly vascularized chorioallantoic membrane (CAM) of chicken embryos. The aim was to assess the inhibitory effect of compound **10e** on neovascularization. In this experiment, CA4P was used as a positive control (Fig. 4**A**). At a concentration 0.5 μM, the reference anti-angiogenic drug, CA4P, elicited 21% inhibition of angiogenesis, but, at this concentration, compound **10e** showed less anti-angiogenic activity (17% inhibition). The anti-angiogenic activity of compound **10e**, however, was comparable with that of CA4P at 5 μM. In this assay, **10e** inhibited blood vessel formation by 51%, compared to 47% inhibition induced by CA4P. At 50 μM, compound **10e** inhibited chicken CAM angiogenesis by 89%, whereas CA4P caused 78% inhibition. Thus, the results showed dose-dependent anti-angiogenic activity for compound **10e** (Fig. 4**B**).

Table 3
Acute toxic effects of heterobivalent β-carbolines in mice and antitumor activities of these compounds against mice bearing Sarcoma 180 and Lewis lung cancer.

Compd.	Mice number		Acute toxicity	Dosage (mg/kg)	Tumor inhibition rate (%)[a]	
	Begin	End	LD_{50} (mg/kg)		Sarcoma 180	Lewis lung cancer
10e	9	9	50	10	46.8 ± 4.7	36.7 ± 5.4
10j	9	9	85	17	37.9 ± 3.9	25.6 ± 4.6
10o	9	8	75	15	33.5 ± 6.7	31.4 ± 3.9
10t	9	9	55	11	39.2 ± 5.5	21.2 ± 4.3
B-9-3[b]			200	40	56.2	40.4
CTX	9	8	–	30	84.2 ± 3.7	81.4 ± 2.3

[a] Data are expressed as mean ± standard deviation.
[b] See Ref. [36].

3. Conclusion

In conclusion, a series of new *N*-acylhydrazone-linked, heterobivalent β-carboline derivatives were synthesized, and their *in vitro* anti-proliferative activities were investigated. SAR studies showed that compound **10e**, containing a 2,3,4,5,6-perfluorophenylmethyl group in the R_9'-position of the β-carboline ring had anti-proliferative activity against EA.HY926 cells, with an IC_{50} value of 2.4 ± 0.8 μM. In addition, this compound exhibited the most potent cytotoxic activity against the tested cancer cell lines, with IC_{50} values ranging from 4.2 ± 0.7 to 18.5 ± 3.1 μM. In mice, compound **10e** exhibited potent antitumor activity, with tumor inhibition of more than 40% for Sarcoma 180. Furthermore, the pharmacological mechanisms showed that compound **10e** has a certain impairment in the motility of LLC cells, which suggests the anti-metastatic potential, and it showed obvious angiogenesis inhibitory effects in CAM assay, and the anti-angiogenetic potency was more potent than the reference drug CA4P at a concentration 50 μM. Compared with the prototype B-9-3, the target compounds didn't exhibit better antitumor activity. So the further pharmacological mechanisms and pharmacological target of this class of compounds are not underway in our laboratory. Preliminary SARs analysis indicated that: the influence on cytotoxic activities of the substituent in the R_9'-position followed the tendency, 2,3,4,5,6-perfluorophenylmethyl > 4-fluorobenzyl > 3-phenylpropyl group.

4. Experimental section

4.1. General information

Column chromatography was performed with silica gel from Huanghai Chemical Reagent (200–300 mesh) and analytical TLC on silica gel 60-F_{254}. Melting points was measured on a WRS-1B melting point apparatus and were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance III HD 400 spectrometer at ambient temperature. Chemical shifts (δ) are reported in ppm relatively to the residual solvent peak. All chemical shifts for ^{1}H and ^{13}C NMR spectroscopy were assigned to residual signals from $CDCl_3$ (^{1}H) at 7.26 ppm and (^{13}C) at 77.16 ppm, or DMSO-d_6 (^{1}H) at 2.50 ppm and (^{13}C) at 39.52 ppm. The multiplicity of each signal is designated by the

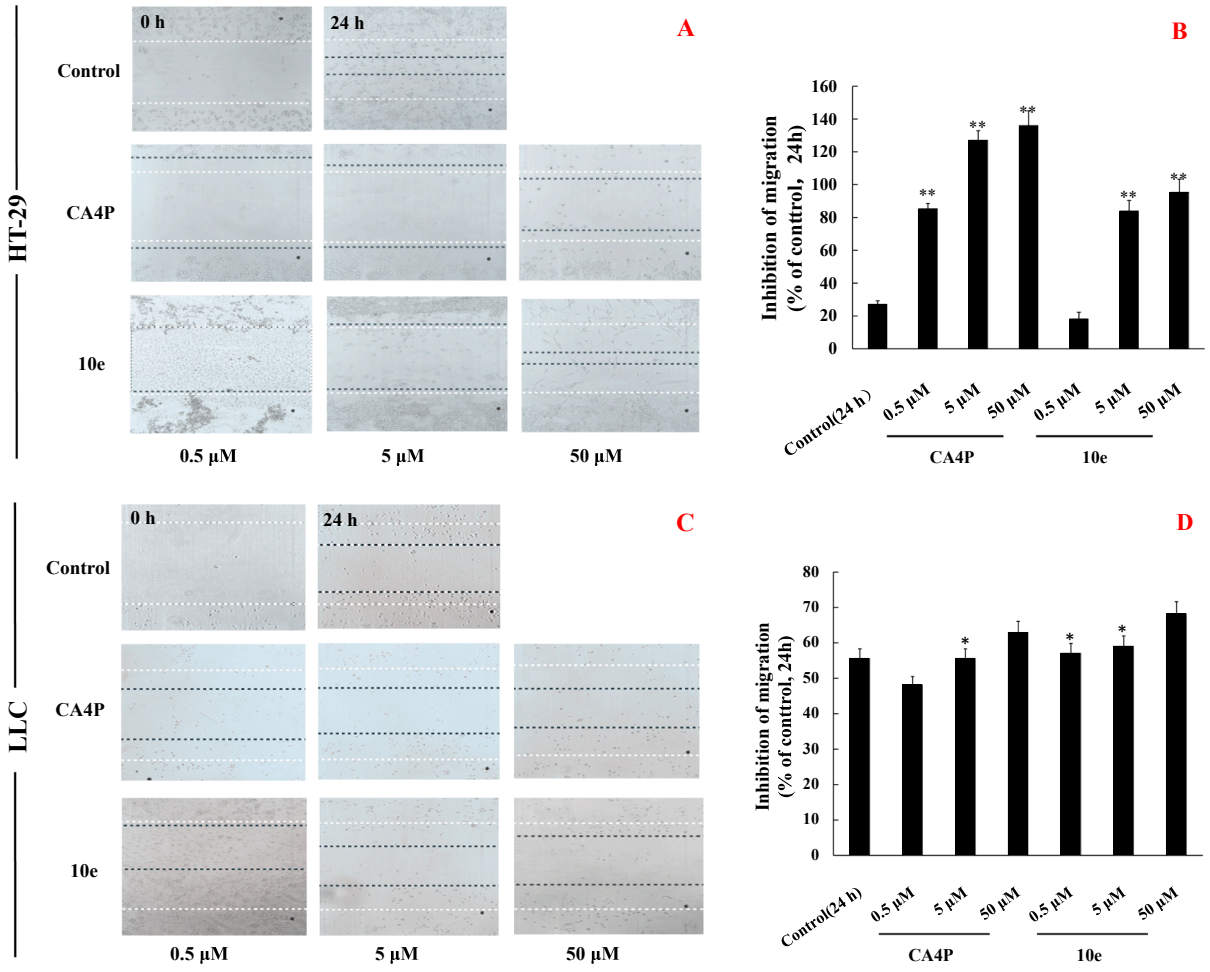


Fig. 3. Wound healing migration assay of HT-29 (A and B) and LLC (C and D) cells after 24 h of treatment with **10e**.

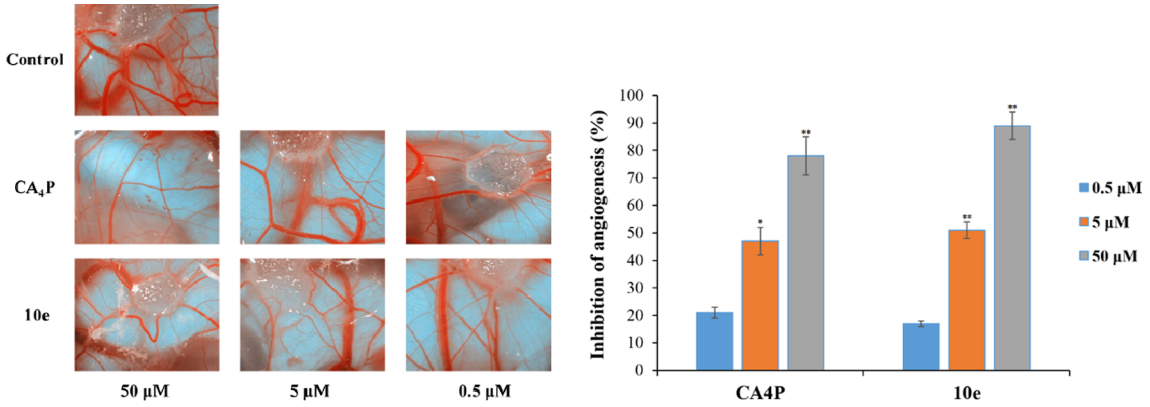


Fig. 4. Inhibitory effects of compound **10e** on the angiogenesis of CAM.

following abbreviations: s = singlet, d = doublet, t = triplet, q = quartet; m = multiplet. Coupling constants (*J*) are quoted in Hz. High resolution mass spectra (HRMS) were recorded on Bruker ultrafleXtreme MALDI-TOF/TOF-MS and Thermo Scientific LTQ Orbitrap XL mass spectrometer.

All reagents and solvents were purchased from commercial suppliers (Adamas-beta, and J&K) and used without further purification. The following intermediates, 9-benzyl-β-carboline-3-carbohydrazide (**6b**) [41], 9-*n*-butyl-1-methyl-β-carboline (**8a**) [41], 9-benzyl-1-methyl-β-carboline (**8b**) [41], 9-(4-fluorobenzyl)-1-methyl-β-carboline (**8c**) [42], 9-(3-phenylpropyl)-1-methyl-β-carboline (**8d**) [41], 9-(2,3,4,5,6-perfluorophenylmethyl)-1-methyl-β-carboline (**8e**) [41], 9-*n*-butyl-β-carboline-1-carboxaldehyde (**9a**) [42], 9-benzyl-β-carboline-1-carboxaldehyde (**9b**) [42], 9-(4-fluorobenzyl)-β-carboline-1-carboxaldehyde (**9c**) [42], 9-(3-phenylpropyl)-β-carboline-1-carboxaldehyde (**9d**) [42] are known compounds.

4.2. General procedure for the preparation of compounds **6a–d**

To a solution of compound **5a–d** (10 mmol) in ethanol (100 mL) was added 85% hydrazine hydrate (10 mL), and then the mixture was refluxed for 8 h. After completion of the reaction as indicated by TLC, the resulting mixture was cooled to 5 ℃, and the precipitate was collected by filtration. The crude product was further purified by washing with ethanol, and by recrystallization in ethanol to obtain compound **6a–d** with yield 80–85%.

4.2.1. 9-n-butyl-β-carboline-3-carbohydrazide (**6a**)

A yellow solid, yield: 94.5%, ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.70 (s, 1H, CO*NH*), 9.05 (d, J = 1.2 Hz, 1H, ArH), 8.84 (d, J = 0.8 Hz, 1H, ArH), 8.44 (d, J = 8.0 Hz, 1H, ArH), 7.77 (d, J = 8.4 Hz, 1H, ArH), 7.68–7.63 (m, 1H, ArH), 7.36–7.31 (m, 1H, ArH), 4.59–4.54 (m, 4H, NH_2, $-CH_2CH_2CH_2CH_3$), 1.85–1.76 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.35–1.24 (m, 2H, $-CH_2CH_2CH_2CH_3$), 0.88 (t, J = 7.2 Hz, 3H, $-CH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.37, 141.62, 140.00, 137.58, 131.70, 129.16, 128.14, 122.87, 121.16, 120.55, 114.14, 110.97, 43.05, 31.38, 20.20, 14.14.

4.2.2. 9-n-butyl-1-methyl-β-carboline-3-carbohydrazide (**6c**)

A light yellow solid, yield: 91.7%, ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.59 (s, 1H, *NH*), 8.67 (s, 1H, ArH), 8.39 (d, J = 7.6 Hz, 1H, ArH), 7.76 (d, J = 8.3 Hz, 1H, ArH), 7.66–7.61 (m, 1H, ArH), 7.34–7.29 (m, 1H, ArH), 4.62 (t, J = 7.6 Hz, 2H, $-CH_2CH_2CH_2CH_3$), 4.57 (s, 2H, NH_2), 3.04 (s, 3H, CH_3), 1.79–1.70 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.44–1.34 (m, 2H, $-CH_2CH_2CH_2CH_3$), 0.92 (t, J = 7.6 Hz, 3H, $-CH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.25, 141.91, 140.62, 138.97, 136.01, 128.98, 128.94, 122.36, 121.30, 120.60, 112.36, 111.11, 44.51, 33.10, 23.64, 20.00, 14.16.

4.2.3. 9-benzyl-1-methyl-β-carboline-3-carbohydrazide (**6d**)

A white solid, yield: 93.8%, ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.57 (s, 1H, *NH*), 8.73 (s, 1H, ArH), 8.46 (d, J = 8.0 Hz, 1H, ArH), 7.72 (d, J = 8.4 Hz, 1H, ArH), 7.62–7.58 (m, 1H, ArH), 7.44–7.16 (m, 4H, ArH), 6.93 (d, J = 7.2 Hz, 2H, ArH), 5.98 (s, 2H, ArCH_2), 4.54 (s, 2H NH_2), 2.85 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.17, 142.41, 140.86, 139.46, 139.13, 136.45, 129.36, 129.25, 127.72, 125.72, 122.54, 121.40, 121.01, 112.51, 111.16, 47.84, 23.24.

4.3. General procedure for the preparation of compounds **9a–e**

To a stirring solution of **8a–e** (5 mmol) in dioxane (100 mL), SeO_2 (10 mmol) was added at room temperature. The reaction medium was kept under stirring and reflux and was monitored by TLC for development of the reactions. After consumption of the starting material, the mixture was cooled and filtered through Celite. The filtrate was evaporated in a vacuum, and the residue was dissolved in EtOAc. The organic layer was washed with water, dried over Na_2SO_4, and evaporated to dryness. The residue was purified by column chromatography (hexane/EtOAc) to afford the compounds **9a–e**.

4.3.1. 9-(2,3,4,5,6-perfluorophenylmethyl)-β-carboline-1-carboxaldehyde (**9e**)

The compound was obtained as a white solid in 89% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.33 (s, 1H, *CH*O), 8.71 (d, J = 4.8 Hz, 1H, ArH), 8.20–8.18 (m, 1H, ArH), 8.15 (d, J = 8.0 Hz, 1H, ArH), 7.62 (t, J = 8.0 Hz, 1H, ArH), 7.46 (d, J = 8.0 Hz, 1H, ArH), 7.38 (t, J = 7.6 Hz, 1H, ArH), 6.54 (s, 2H, ArCH_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.4, 146.5 (m), 144.0 (m), 142.1 (m), 141.4, 139.2, 138.8 (m), 138.2, 136.3 (m), 136.2, 132. 6, 129. 8, 121.6, 121.6, 121.4 118.8, 110.8 (m), 109. 9 (t, J = 2.1 Hz), 39.6.

4.4. General procedure for the preparation of heterobivalent β-carbolines 10a-t

To a solution of β-carboline-3-carbohydrazide **6a–d** (1 mmol) in ethanol (30 mL) was added the corresponding β-carboline-1-carbaldehyde **9a–e** (1 mmol), then reaction mixture was refluxed for 5 h. After completion of the reaction as indicated by TLC, the solution was allowed to cool to room temperature. Then the precipitates formed and filtered, the crude product was recrystallized with ethanol to afford the target compounds **10a–t**.

4.4.1. N'-((9-n-butyl-9H-pyrido[3,4-b]indol-1-yl)methylene)-9-n-butyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10a**)

A light yellow solid, yield: 92.4%, m.p. 213.2–214.4℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.61 (s, 1H, *NH*), 9.18 (d, J = 3.2 Hz, 2H, ArH), 9.06 (d, J = 0.8 Hz, 1H, ArH), 8.52–8.49 (m, 2H, ArH), 8.35 (d,

J = 8.0 Hz, 1H, ArH), 8.29 (d, *J* = 4.8 Hz, 1H, *CH* = N), 7.86 (d, *J* = 8.8 Hz, 1H, ArH), 7.83 (d, *J* = 8.4 Hz, 1H, ArH), 7.72–7.65 (m, 2H, ArH), 7.41–7.32 (m, 2H, ArH), 5.05 (t, *J* = 7.6 Hz, 2H, $-CH_2CH_2CH_2CH_3$), 4.65 (t, *J* = 8.2 Hz, 2H, $-CH_2CH_2CH_2CH_3$), 1.89–1.81 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.56–1.48 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.36–1.27 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.16–1.06 (m, 2H, $-CH_2CH_2CH_2CH_3$), 0.90 (t, *J* = 7.2 Hz, 3H, $-CH_2CH_2CH_2CH_3$), 0.75 (t, *J* = 7.2 Hz, 3H, $-CH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, DMSO-d_6) *δ* 162.25, 148.15, 142.71, 141.76, 139.43, 138.43, 138.40, 138.03, 133.85, 131.66, 131.28, 129.40, 129.24, 128.44, 123.03, 122.07, 121.18, 120.96, 120.84, 120.47, 115.79, 115.69, 111.38, 111.16, 44.67, 43.14, 31.42, 31.09, 20.22, 19.73, 14.18, 14.08. HRMS calcd for $C_{32}H_{33}N_6O$ $[M+H]^+$ 517.2710, found 517.2712.

*4.4.2. N'-((9-benzyl-9H-pyrido[3,4-b]indol-1-yl)methylene)-9-n-butyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10b**)*

A light yellow solid, yield: 90.3%, m.p. 211.2–213.5℃. ^{1}H NMR (400 MHz, DMSO-d_6) *δ* 12.59 (s, 1H, *NH*), 9.18 (d, *J* = 0.8 Hz, 1H, ArH), 9.10 (d, *J* = 0.8 Hz, 1H, ArH), 8.94 (s, 1H, ArH), 8.55–8.49 (m, 2H, ArH), 8.41 (d, *J* = 7.6 Hz, 1H, ArH), 8.34 (d, *J* = 4.8 Hz, 1H, *CH* = N), 8.00 (d, *J* = 8.4 Hz, 1H, ArH), 7.83 (d, *J* = 8.4 Hz, 1H, ArH), 7.73–7.67 (m, 2H, ArH), 7.39 (t, *J* = 7.6 Hz, 2H, ArH), 6.97–6.91 (m, 2H, ArH), 6.77–6.72 (m, 2H, ArH), 6.52 (s, 2H, ArCH_2), 4.65 (t, *J* = 6.8 Hz, 2H, $-CH_2CH_2CH_2CH_3$), 1.89–1.81 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.37–1.26 (m, 2H, $-CH_2CH_2CH_2CH_3$), 0.90 (t, *J* = 7.2 Hz, 3H, $-CH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, DMSO-d_6) *δ* 162.51, 148.50, 143.14, 141.77, 139.33, 138.95, 138.70, 138.06, 135.12, 135.09, 133.92, 131.75, 131.71, 129.60, 129.41, 128.43, 128.18, 128.10, 123.02, 122.20, 121.19, 121.02, 120.87, 115.96, 115.84, 115.80, 115.63, 111.64, 111.18, 47.68, 43.14, 31.42, 20.21, 14.17. HRMS calcd for $C_{35}H_{31}N_6O$ $[M+H]^+$ 551.2554, found 551.2553.

*4.4.3. N'-((9-(4-fluorobenzyl)-9H-pyrido[3,4-b]indol-1-yl)methylene)-9-n-butyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10c**)*

A light yellow solid, yield: 84.9%, m.p. 250.6–252.3℃. ^{1}H NMR (400 MHz, DMSO-d_6) *δ* 12.57 (d, *J* = 5.6 Hz, 1H, *NH*), 9.18 (s, 1H, ArH), 9.09 (s, 1H, ArH), 8.93 (s, 1H, ArH), 8.55–8.49 (m, 2H, ArH), 8.40 (d, *J* = 7.6 Hz, 1H, ArH), 8.34 (d, *J* = 4.8 Hz, 1H, *CH* = N), 7.99 (d, *J* = 8.4 Hz, 1H, ArH), 7.83 (d, *J* = 8.4 Hz, 1H, ArH), 7.73–7.67 (m, 2H, ArH), 7.39 (t, *J* = 7.6 Hz, 2H, ArH), 7.11–7.06 (m, 1H, ArH), 6.97–6.89 (m, 2H, ArH), 6.76–6.69 (m, 2H, ArH), 6.52 (s, 2H, ArCH_2), 4.65 (t, *J* = 6.8 Hz, 2H, $-CH_2CH_2CH_2CH_3$), 1.89–1.81 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.37–1.26 (m, 2H, $-CH_2CH_2CH_2CH_3$), 0.90 (t, *J* = 7.6 Hz, 3H, $-CH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, DMSO-d_6) *δ* 162.50, 161.45 (d, *J* = 245 Hz), 148.48, 143.14, 141.77, 139.32, 138.95, 138.69, 138.06, 135.11, 133.92, 131.76 (d, *J* = 4.4 Hz), 129.61, 129.42, 128.89, 128.43, 128.18 (d, *J* = 8.1 Hz), 126.22, 123.02, 122.21, 121.19, 121.03, 120.87, 115.97, 115.80, 115.63 (d, *J* = 21.2 Hz), 111.64, 111.19, 47.68, 43.15, 31.43, 20.22, 14.18. HRMS calcd for $C_{35}H_{30}FN_6O$ $[M+H]^+$ 569.2460, found 569.2459.

*4.4.4. N'-((9-(3-phenylpropyl)-9H-pyrido[3,4-b]indol-1-yl)methylene)-9-n-butyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10d**)*

A light yellow solid, yield: 83.6%, m.p. 233.4–235.1℃. ^{1}H NMR (400 MHz, DMSO-d_6) *δ* 12.62 (s, 1H, *NH*), 9.18 (d, *J* = 2.4 Hz, 2H, ArH), 9.07 (s, 1H, ArH), 8.52–8.49 (m, 2H, ArH), 8.35 (d, *J* = 7.6 Hz, 1H, ArH), 8.29 (d, *J* = 5.2 Hz, 1H, *CH* = N), 7.83 (d, *J* = 8.4 Hz, 1H, ArH), 7.75–7.63 (m, 3H, ArH), 7.40–7.32 (m, 2H, ArH), 7.15–7.10 (m, 2H, ArH), 7.06–7.02 (m, 3H, ArH), 5.11 (t, *J* = 7.6 Hz, 2H, $ArCH_2CH_2CH_2$), 4.65 (t, *J* = 6.8 Hz, 2H, $-CH_2CH_2CH_2CH_3$), 2.51–2.46 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.90–1.81 (m, 4H, $-CH_2CH_2CH_2CH_3$, $ArCH_2CH_2CH_2$), 1.36–1.26 (m, 2H, $ArCH_2CH_2CH_2$), 0.89 (t, *J* = 7.2 Hz, 3H, $-CH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, DMSO-d_6) *δ* 162.30, 148.40, 142.55, 141.76, 139.46, 138.51, 138.35, 138.05, 133.86, 131.67, 131.30, 129.40, 129.27, 128.65, 128.47, 128.44, 126.14, 123.05, 122.15, 122.11, 121.19, 120.98, 120.86, 120.55, 115.84, 115.72, 111.22, 111.18, 43.14, 32.53, 31.42, 31.09, 20.22, 14.18. HRMS calcd for $C_{37}H_{35}N_6O$ $[M+H]^+$ 579.2867, found 579.2864.

*4.4.5. N'-((9-((perfluorophenyl)methyl)-9H-pyrido[3,4-b]indol-1-yl)methylene)-9-n-butyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10e**)*

A light yellow solid, yield: 88.1%, m.p. 258.6–260.9℃. ^{1}H NMR (400 MHz, DMSO-d_6) *δ* 12.61 (s, 1H, *NH*), 9.16 (d, *J* = 1.2 Hz, 1H, ArH), 9.05 (s, 1H, ArH), 9.02 (d, *J* = 0.8 Hz, 1H, ArH), 8.55 (d, *J* = 4.8 Hz, 1H, ArH), 8.49 (d, *J* = 8.0 Hz, 1H, ArH), 8.37 (d, *J* = 7.6 Hz, 1H, ArH), 8.31 (d, *J* = 4.8 Hz, 1H, *CH* = N), 7.82 (d, *J* = 8.4 Hz, 1H, ArH), 7.79 (d, *J* = 8.4 Hz, 1H, ArH), 7.72–7.66 (m, 2H, ArH), 7.41–7.35 (m, 2H, ArH), 6.76 (s, 2H, ArCH_2), 4.64 (t, *J* = 6.8 Hz, 2H, $-CH_2CH_2CH_2CH_3$), 1.89–1.81 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.36–1.26 (m, 2H, $-CH_2CH_2CH_2CH_3$), 0.89 (t, *J* = 7.2 Hz, 3H, $-CH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, DMSO-d_6) *δ* 162.36, 149.16, 146.39 (m), 143.94 (m), 142.58, 142.58, 141.74, 139.35, 139.29, 139.07, 138.02, 136.08 (m), 134.56, 131.85, 131.66, 129.59, 129.40, 128.39, 123.00, 122.20, 121.58, 121.36, 121.16, 120.84, 115.90, 115.69, 111.80 (m), 111.17, 43.13, 31.41, 20.21, 14.16. HRMS calcd for $C_{35}H_{26}F_5N_6O$ $[M+H]^+$ 641.2083, found 641.2081.

*4.4.6. N'-((9-n-butyl-9H-pyrido[3,4-b]indol-1-yl)methylene)-9-benzyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10f**)*

A light yellow solid, yield: 89.7%, m.p. 237.2–238.9℃. ^{1}H NMR (400 MHz, DMSO-d_6) *δ* 12.60 (s, 1H, *NH*), 9.21 (d, *J* = 0.8 Hz, 1H, ArH), 9.17 (s, 1H, ArH), 9.08 (d, *J* = 0.8 Hz, 1H, ArH), 8.53 (d, *J* = 7.6 Hz, 1H, ArH), 8.50 (d, *J* = 4.8 Hz, 1H, ArH), 8.34 (d, *J* = 7.6 Hz, 1H, ArH), 8.28 (d, *J* = 4.8 Hz, 1H, *CH* = N), 7.85 (dd, *J* = 8.4, 4.0 Hz, 2H, ArH), 7.71–7.65 (m, 2H, ArH), 7.42–7.38 (m, 1H, ArH), 7.34–7.26 (m, 7H, ArH), 5.93 (s, 2H, ArCH_2), 5.04 (t, *J* = 7.2 Hz, 2H, $-CH_2CH_2CH_2CH_3$), 1.55–1.47 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.15–1.05 (m, 2H, $-CH_2CH_2CH_2CH_3$), 0.74 (t, *J* = 7.2 Hz, 3H, $-CH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, DMSO-d_6) *δ* 162.19, 148.19, 142.71, 141.90, 139.84, 138.43, 138.37, 138.13, 137.59, 133.85, 131.95, 131.28, 129.58, 129.24, 128.82, 128.10, 127.42, 127.29, 123.13, 122.07, 121.39, 121.16, 120.96, 120.47, 115.80, 115.76, 111.40, 46.74, 44.66, 31.08, 19.73, 14.08. HRMS calcd for $C_{35}H_{31}N_6O$ $[M+H]^+$ 551.2554, found 551.2553.

*4.4.7. N'-((9-benzyl-9H-pyrido[3,4-b]indol-1-yl)methylene)-9-benzyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10g**)*

A light yellow solid, yield: 88.8%, m.p. 250.3–251.3℃. ^{1}H NMR (400 MHz, DMSO-d_6) *δ* 12.56 (s, 1H, *NH*), 9.21 (d, *J* = 1.2 Hz, 1H, ArH), 9.12 (d, *J* = 1.2 Hz, 1H, ArH), 8.91 (s, 1H, ArH), 8.54 (d, *J* = 8.0 Hz, 1H, ArH), 8.51 (d, *J* = 4.8 Hz, 1H, ArH), 8.40 (d, *J* = 7.6 Hz, 1H, ArH), 8.33 (d, *J* = 4.8 Hz, 1H, *CH* = N), 7.99 (d, *J* = 8.4 Hz, 1H, ArH), 7.85 (d, *J* = 8.4 Hz, 1H, ArH), 7.71–7.66 (m, 2H, ArH), 7.40 (q, *J* = 7.2 Hz, 2H, ArH), 7.32–7.25 (m, 5H, ArH), 7.10–7.05 (m, 3H, ArH), 6.71–6.68 (m, 2H, ArH), 6.51 (s, 2H, ArCH_2), 5.93 (s, 2H, ArCH_2). ^{13}C NMR (100 MHz, DMSO-d_6) *δ* 162.40, 148.41, 143.27, 141.90, 139.77, 138.92, 138.86, 138.70, 138.16, 137.59, 134.05, 131.98, 131.68, 129.59, 129.55, 129.24, 128.89, 128.82, 128.11, 127.42, 127.27, 126.22, 123.12, 122.18, 121.40, 121.18, 121.15, 120.95, 115.93, 115.85, 111.67, 111.42, 48.28, 46.74. HRMS calcd for $C_{38}H_{29}N_6O$ $[M+H]^+$ 585.2397, found 585.2399.

*4.4.8. N'-((9-(4-fluorobenzyl)-9H-pyrido[3,4-b]indol-1-yl)methylene)-9-benzyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10h**)*

A light yellow solid, yield: 82.4%, m.p. 270.6–272.2℃. ^{1}H NMR (400 MHz, DMSO-d_6) *δ* 12.58 (s, 1H, *NH*), 9.21 (d, *J* = 1.2 Hz, 1H, ArH), 9.12 (d, *J* = 1.2 Hz, 1H, ArH), 8.91 (s, 1H, ArH), 8.54 (d, *J* = 8.0 Hz, 1H, ArH), 8.52 (d, *J* = 4.8 Hz, 1H, ArH), 8.40 (d, *J* = 7.2 Hz, 1H, ArH), 8.33 (d, *J* = 4.8 Hz, 1H, *CH* = N), 7.99 (d, *J* = 8.4 Hz, 1H, ArH), 7.86 (d, *J* = 8.4 Hz, 1H, ArH), 7.72–7.66 (m, 2H, ArH), 7.43–7.37 (m, 2H, ArH), 7.34–7.25 (m, 5H, ArH), 6.96–6.91 (m,

2H, ArH), 6.75–6.71 (m, 2H, ArH), 6.51 (s, 2H, ArCH_2), 5.93 (s, 2H, ArCH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.44, 161.44 (d, J = 241.1 Hz), 148.54, 143.15, 141.90, 139.74, 138.95, 138.68, 138.16, 137.59, 135.08 (d, J = 2.9 Hz), 133.91, 131.99, 131.76, 129.61, 129.24, 128.81, 128.18 (d, J = 7.7 Hz), 127.42, 123.12, 122.21, 121.40, 121.18, 121.03, 115.97, 115.87, 115.84 (d, J = 21.3 Hz), 111.64, 111.43, 47.67, 46.74. HRMS calcd for $C_{38}H_{28}FN_6O$ $[M+H]^+$ 603.2303, found 603.2309.

4.4.9. N'-((9-(3-phenylpropyl)-9H-pyrido[3,4-b]indol-1-yl)methylene)-9-benzyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10i**)

A light yellow solid, yield: 83.1%, m.p. 233.8–235.9℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.62 (s, 1H, *NH*), 9.22 (d, J = 0.8 Hz, 1H, ArH), 9.16 (s, 1H, ArH), 9.09 (d, J = 0.8 Hz, 1H, ArH), 8.54 (d, J = 7.6 Hz, 1H, ArH), 8.51 (d, J = 4.8 Hz, 1H, ArH), 8.34 (d, J = 7.6 Hz, 1H, ArH), 8.29 (d, J = 5.2 Hz, 1H, *CH* = N), 7.85 (d, J = 8.4 Hz, 1H, ArH), 7.73 (d, J = 8.4 Hz, 1H, ArH), 7.70–7.64 (m, 2H, ArH), 7.42–7.37 (m, 1H, ArH), 7.34–7.25 (m, 6H, ArH), 7.14–7.09 (m, 2H, ArH), 7.06–7.01 (m, 3H, ArH), 5.93 (s, 2H, ArCH_2), 5.10 (t, J = 7.6 Hz, 2H, Ar$CH_2CH_2CH_2$), 2.47 (t, J = 8.0 Hz, 2H, Ar$CH_2CH_2CH_2$), 1.89–1.80 (m, 2H, Ar$CH_2CH_2CH_2$). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.24, 148.43, 142.54, 141.91, 141.74, 139.87, 138.50, 138.31, 138.14, 137.59, 133.86, 131.96, 131.30, 129.58, 129.27, 129.24, 128.83, 128.64, 128.47, 128.10, 127.41, 126.14, 123.14, 122.11, 121.40, 121.17, 120.98, 120.54, 115.85, 115.77, 111.41, 111.21, 46.74, 44.84, 32.53, 31.08. HRMS calcd for $C_{40}H_{33}N_6O$ $[M+H]^+$ 613.2710, found 613.2712.

4.4.10. N'-((9-((perfluorophenyl)methyl)-9H-pyrido[3,4-b]indol-1-yl)methylene)-9-benzyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10j**)

A light yellow solid, yield: 84.7%, m.p. 258.4–260.1℃. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.60 (s, 1H, *NH*), 9.20 (d, J = 0.8 Hz, 1H, ArH), 9.04 (d, J = 0.8 Hz, 1H, ArH), 9.03 (s, 1H, ArH), 8.54 (d, J = 5.2 Hz, 1H, ArH), 8.52 (d, J = 8.0 Hz, 1H, ArH), 8.36 (d, J = 7.6 Hz, 1H, ArH), 8.30 (d, J = 4.8 Hz, 1H, *CH* = N), 7.84 (d, J = 8.4 Hz, 1H, ArH), 7.78 (d, J = 8.4 Hz, 1H, ArH), 7.71–7.65 (m, 2H, ArH), 7.39 (td, J = 7.6, 2.4 Hz, 2H, ArH), 7.34–7.29 (m, 2H, ArH), 7.29–7.24 (m, 3H, ArH), 6.75 (s, 2H, ArCH_2), 5.92 (s, 2H, ArCH_2). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.30, 149.21, 146.49 (m), 143.92 (m), 142.57, 141.88, 139.70, 139.35, 139.04, 138.13, 137.57, 136.10 (m), 134.56, 131.95, 131.85, 129.59, 129.23, 128.78, 128.10, 127.41, 123.10, 122.20, 121.58, 121.37, 121.16, 115.91, 115.76, 111.78 (m), 111.41, 111.18, 46.74. HRMS calcd for $C_{38}H_{24}F_5N_6O$ $[M+H]^+$ 675.1926, found 675.1924.

4.4.11. N'-((9-n-butyl-9H-pyrido[3,4-b]indol-1-yl)methylene)-1-methyl-9-n-butyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10k**)

A light yellow solid, yield: 86.3%, m.p. 199.7–201.3℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.46 (s, 1H, *NH*), 9.08 (s, 1H, ArH), 8.90 (s, 1H, ArH), 8.54 (d, J = 4.8 Hz, 1H, ArH), 8.21–8.14 (m, 2H, ArH), 8.03 (d, J = 4.8 Hz, 1H, *CH* = N), 7.65–7.57 (m, 3H, ArH), 7.49 (d, J = 8.4 Hz, 1H, ArH), 7.36–7.29 (m, 2H, ArH), 5.08 (t, J = 7.2 Hz, 2H, $-CH_2CH_2CH_2CH_3$), 4.57 (t, J = 7.6 Hz, 2H, $-CH_2CH_2CH_2CH_3$), 3.10 (s, 3H, CH_3), 1.90–1.82 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.74–1.63 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.53–1.43 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.31–1.20 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.00 (t, J = 7.2 Hz, 3H, $-CH_2CH_2CH_2CH_3$), 0.84 (t, J = 7.2 Hz, 3H, $-CH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.56, 147.73, 142.79, 141.84, 139.79, 138.13, 137.65, 137.10, 136.67, 134.52, 131.72, 129.62, 128.67, 128.62, 121.90, 121.66, 121.26, 121.10, 120.63, 119.95, 115.30, 113.72, 110.58, 110.03, 45.22, 44.88, 33.03, 31.40, 23.75, 20.22, 19.96, 13.92, 13.88. HRMS calcd for $C_{33}H_{35}N_6O$ $[M+H]^+$ 531.2867, found 531.2873.

4.4.12. N'-((9-benzyl-9H-pyrido[3,4-b]indol-1-yl)methylene)-1-methyl-9-n-butyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10l**)

A light yellow solid, yield: 90.8%, m.p. 246.8–248.2℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.29 (s, 1H, *NH*), 8.92 (d, J = 2.0 Hz, 1H, ArH), 8.53 (s, 1H, ArH), 8.51 (dd, J = 5.2, 1.6 Hz, 1H, ArH), 8.19 (d, J = 7.6 Hz, 2H, ArH, *CH* = N), 8.04–8.01 (m, 1H, ArH), 7.62–7.58 (m, 3H, ArH), 7.48–7.45 (m, 1H, ArH), 7.37–7.30 (m, 3H, ArH), 7.09–7.06 (m, 3H, ArH), 6.82–6.79 (m, 2H, ArH), 6.43 (s, 2H, ArCH_2), 4.51 (t, J = 7.2 Hz, 2H, $-CH_2CH_2CH_2CH_3$), 3.05 (s, 3H, CH_3), 1.87–1.80 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.50–1.41 (m, 2H, $-CH_2CH_2CH_2CH_3$), 0.99 (t, J = 7.2 Hz, 3H, $-CH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.54, 147.15, 143.22, 141.79, 139.77, 138.45, 138.10, 137.49, 137.43, 136.62, 134.49, 131.86, 129.29, 129.06, 128.60, 128.46, 126.83, 125.96, 121.84, 121.61, 121.34, 121.12, 120.61, 120.41, 115.25, 113.73, 110.56, 110.03, 48.69, 44.82, 33.01, 23.67, 20.19, 13.86. ^{19}F NMR (376 MHz, $CDCl_3$) δ −115.94. HRMS calcd for $C_{36}H_{33}N_6O$ $[M+H]^+$ 565.2710, found 565.2703.

4.4.13. N'-((9-(4-fluorobenzyl)-9H-pyrido[3,4-b]indol-1-yl)methylene)-1-methyl-9-n-butyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10m**)

A light yellow solid, yield: 95.7%, m.p. 264.8–267.1℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.31 (s, 1H, *NH*), 8.91 (s, 1H, ArH), 8.55 (s, 1H, ArH), 8.51 (d, J = 5.2 Hz, 1H, ArH), 8.19 (dd, J = 8.0, 4.8 Hz, 2H, ArH), 8.02 (d, J = 5.2 Hz, 1H, *CH* = N), 7.64–7.57 (m, 3H, ArH), 7.47 (d, J = 8.4 Hz, 1H, ArH), 7.38–7.32 (m, 2H, ArH), 6.81–6.73 (m, 4H, ArH), 6.44 (s, 2H, ArCH_2), 4.51 (t, J = 7.6 Hz, 2H, $-CH_2CH_2CH_2CH_3$), 3.06 (s, 3H, CH_3), 1.88–1.80 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.51–1.41 (m, 2H, $-CH_2CH_2CH_2CH_3$), 0.99 (t, J = 7.2 Hz, 3H, $-CH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.60, 160.46 (d, J = 243.1 Hz), 147.47, 143.09, 141.80, 139.83, 138.58, 137.42, 136.64, 134.32, 133.89 (d, J = 3.0 Hz), 131.91, 129.51, 129.11, 128.63, 127.65 (d, J = 8.0 Hz), 121.83, 121.60, 121.37, 121.16, 120.64, 120.52, 115.39 (d, J = 21.3 Hz), 115.29, 113.72, 110.49, 110.05, 48.12, 44.82, 33.01, 23.68, 20.19, 13.85. HRMS calcd for $C_{36}H_{32}FN_6O$ $[M+H]^+$ 583.2616, found 583.2621.

4.4.14. N'-((9-(3-phenylpropyl)-9H-pyrido[3,4-b]indol-1-yl)methylene)-1-methyl-9-n-butyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10n**)

A light yellow solid, yield: 88.4%, m.p. 203.2–205.5℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.41 (s, 1H, *NH*), 8.98 (s, 1H, ArH), 8.91 (s, 1H, ArH), 8.54 (d, J = 5.2 Hz, 1H, ArH), 8.20 (d, J = 8.0 Hz, 1H, ArH), 8.13 (d, J = 8.0 Hz, 1H, ArH), 8.01 (d, J = 5.2 Hz, 1H, *CH* = N), 7.64–7.56 (m, 2H, ArH), 7.49 (d, J = 8.4 Hz, 1H, ArH), 7.42 (d, J = 8.4 Hz, 1H, ArH), 7.36–7.27 (m, 2H, ArH), 7.19–7.14 (m, 2H, ArH), 7.09–7.05 (m, 3H, ArH), 5.14 (t, J = 7.6 Hz, 2H, Ar$CH_2CH_2CH_2$), 4.56 (t, J = 7.6 Hz, 2H, $-CH_2CH_2CH_2CH_3$), 3.10 (s, 3H, CH_3), 2.60 (t, J = 7.6 Hz, 2H, Ar$CH_2CH_2CH_2$), 2.08–2.01 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.90–1.82 (m, 2H, Ar$CH_2CH_2CH_2$), 1.52–1.42 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.00 (t, J = 7.6 Hz, 3H, $-CH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.54, 147.89, 142.62, 141.83, 141.41, 139.77, 138.21, 137.64, 137.11, 136.65, 134.48, 131.76, 129.61, 128.70, 128.61, 128.35, 128.25, 125.77, 121.89, 121.65, 121.23, 121.12, 120.62, 120.02, 115.30, 113.72, 110.55, 110.03, 44.96, 44.87, 33.02, 32.64, 30.72, 23.73, 20.21, 13.87. HRMS calcd for $C_{38}H_{37}N_6O$ $[M+H]^+$ 593.3023, found 593.3019.

4.4.15. N'-((9-((perfluorophenyl)methyl)-9H-pyrido[3,4-b]indol-1-yl)methylene)-1-methyl-9-n-butyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10o**)

A white solid, yield: 85.9%, m.p. 253.6–255.1℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.43 (s, 1H, *NH*), 8.85 (s, 1H, ArH), 8.78 (s, 1H, ArH), 8.56 (d, J = 5.2 Hz, 1H, ArH), 8.17 (d, J = 8.0 Hz, 1H, ArH), 8.12 (d, J = 7.6 Hz, 1H, ArH), 7.99 (d, J = 4.8 Hz, 1H, *CH* = N), 7.63–7.55 (m, 3H, ArH), 7.48 (d, J = 8.4 Hz, 1H, ArH), 7.33 (t, J = 7.2 Hz, 2H, ArH), 6.89 (s, 2H, ArCH_2), 4.54 (t, J = 7.6 Hz, 2H, $-CH_2CH_2CH_2CH_3$), 3.08 (s, 3H, CH_3), 1.88–1.80 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.52–1.42 (m, 2H, $-CH_2CH_2CH_2CH_3$), 0.99 (t, J = 7.6 Hz, 3H, $-CH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.58, 149.27, 146.64 (m), 144.19 (m), 142.00, 141.82, 139.88,

138.85, 138.71 (m), 138.04, 137.33, 136.68, 136.20 (m), 135.44, 132.03, 129.55, 129.15, 128.66, 121.85, 121.80, 121.61, 121.28, 120.99, 120.67, 115.34, 113.75, 111.47 (m), 110.27, 110.04, 44.87, 39.06, 33.02, 23.69, 20.20, 13.86. HRMS calcd for $C_{36}H_{28}F_5N_6O$ $[M+H]^+$ 655.2239, found 655.2244.

4.4.16. N'-((9-n-butyl-9H-pyrido[3,4-b]indol-1-yl)methylene)-1-methyl-9-benzyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10p**)

A light yellow solid, yield: 83.3%, m.p. 213.5–215.1℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.42 (s, 1H, *NH*), 9.04 (s, 1H, ArH), 8.94 (s, 1H, ArH), 8.53 (d, J = 5.2 Hz, 1H, ArH), 8.22 (d, J = 7.6 Hz, 1H, ArH), 8.13 (d, J = 7.6 Hz, 1H, ArH), 8.00 (d, J = 5.2 Hz, 1H, CH), 7.63–7.53 (m, 3H, ArH), 7.41–7.23 (m, 6H, ArH), 6.98–6.95 (m, 2H, ArH), 5.81 (s, 2H, ArCH_2), 5.07 (t, J = 7.6 Hz, 2H, $-CH_2CH_2CH_2CH_3$), 2.91 (s, 3H, CH_3), 1.71–1.63 (m, 2H, $-CH_2CH_2CH_2CH_3$), 1.30–1.20 (m, 2H, $-CH_2CH_2CH_2CH_3$), 0.83 (t, J = 7.6 Hz, 3H, $-CH_2CH_2CH_2CH_3$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.44, 147.79, 142.75, 142.24, 140.26, 138.12, 137.44, 137.05, 137.01, 134.49, 131.69, 129.74, 129.11, 129.06, 128.96, 128.68, 127.72, 125.29, 121.88, 121.64, 121.25, 121.06, 121.04, 119.95, 115.29, 113.70, 110.55, 110.15, 48.27, 45.20, 31.39, 23.31, 19.94, 13.91. HRMS calcd for $C_{36}H_{33}N_6O$ $[M+H]^+$ 565.2710, found 565.2704.

4.4.17. N'-((9-benzyl-9H-pyrido[3,4-b]indol-1-yl)methylene)-1-methyl-9-benzyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10q**)

A light yellow solid, yield: 89.5%, m.p. 247.7–249.5℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.27 (s, 1H, *NH*), 9.00 (s, 1H, ArH), 8.52 (d, J = 5.2 Hz, 2H, ArH), 8.27 (d, J = 7.2 Hz, 1H, ArH), 8.21 (d, J = 8.0 Hz, 1H, ArH), 8.06 (d, J = 5.2 Hz, 1H, *CH* = N), 7.64–7.56 (m, 3H, ArH), 7.44–7.35 (m, 3H, ArH), 7.32–7.26 (m, 3H, ArH), 7.08– 7.06 (m, 3H, ArH), 7.01–6.97 (m, 2H, ArH), 6.81–6.77 (m, 2H, ArH), 6.44 (s, 2H, ArCH_2), 5.85 (s, 2H, ArCH_2), 2.91 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.48, 147.13, 143.31, 142.30, 140.30, 138.33, 138.06, 138.04, 137.43, 137.36, 137.16, 134.48, 132.00, 129.80, 129.15, 129.10, 129.02, 128.47, 127.76, 126.85, 125.95, 125.32, 121.97, 121.71, 121.39, 121.12, 120.48, 115.32, 113.86, 110.61, 110.19, 48.71, 48.33, 23.31. HRMS calcd for $C_{39}H_{31}N_6O$ $[M+H]^+$ 599.2554, found 599.2563.

4.4.18. N'-((9-(4-fluorobenzyl)-9H-pyrido[3,4-b]indol-1-yl)methylene)-1-methyl-9-benzyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10r**)

A light yellow solid, yield: 91.6%, m.p. > 280℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.29 (s, 1H, *NH*), 8.98 (s, 1H, ArH), 8.53 (s, 1H, ArH), 8.51 (d, J = 5.2 Hz, 1H, ArH), 8.26 (d, J = 7.6 Hz, 1H, ArH), 8.20 (d, J = 8.0 Hz, 1H, ArH), 8.04 (d, J = 5.2 Hz, 1H, *CH* = N), 7.66–7.55 (m, 3H, ArH), 7.42–7.34 (m, 3H, ArH), 7.31–7.27 (m, 3H, ArH), 6.99–6.96 (m, 2H, ArH), 6.79–6.72 (m, 4H, ArH), 6.44 (s, 2H, ArCH_2), 5.82 (s, 2H, ArCH_2), 2.90 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.53, 160.47 (d, J = 243.4 Hz), 147.44, 143.15, 142.28, 140.35, 138.48, 137.95, 137.40, 137.36, 137.13, 134.31, 133.86 (d, J = 2.9 Hz), 132.02, 129.75, 129.19, 129.14, 129.03, 127.76, 127.63 (d, J = 8.0 Hz), 125.30, 121.93, 121.66, 121.41, 121.15, 121.11, 120.57, 115.41 (d, J = 21.3 Hz), 115.34, 113.81, 110.52, 110.20, 48.30, 48.13, 23.30. ^{19}F NMR (376 MHz, $CDCl_3$) δ −115.92. HRMS calcd for $C_{39}H_{30}FN_6O$ $[M+H]^+$ 617.2460, found 617.2455.

4.4.19. N'-((9-(3-phenylpropyl)-9H-pyrido[3,4-b]indol-1-yl)methylene)-1-methyl-9-benzyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10s**)

A yellow solid, yield: 87.2%, m.p. 214.8–216.2℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.38 (s, 1H, *NH*), 8.96 (s, 2H, ArH), 8.53 (d, J = 4.8 Hz, 1H, ArH), 8.25 (d, J = 7.6 Hz, 1H, ArH), 8.13 (d, J = 7.6 Hz, 1H, ArH), 8.01 (d, J = 5.2 Hz, 1H, *CH* = N), 7.61–7.54 (m, 2H, ArH), 7.44–7.35 (m, 3H, ArH), 7.32–7.26 (m, 4H, ArH), 7.18–7.14 (m, 2H, ArH), 7.08–7.04 (m, 3H, ArH), 7.00–6.96 (m, 2H, ArH), 5.84 (s, 2H, ArCH_2), 5.13 (t, J = 7.6 Hz, 2H, $ArCH_2CH_2CH_2$), 2.93 (s, 3H, CH_3), 2.59 (t, J = 7.6 Hz, 2H, $ArCH_2CH_2CH_2$), 2.07–2.00 (m, 2H, $ArCH_2CH_2CH_2$). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.46, 147.91, 142.63, 142.28, 141.39, 140.27, 138.16, 137.44, 137.10, 137.02, 134.46, 131.79, 129.80, 129.14, 128.99, 128.73, 128.34, 128.26, 127.74, 125.77, 125.30, 121.95, 121.69, 121.25, 121.11, 121.07, 120.05, 115.32, 113.77, 110.55, 110.16, 48.31, 44.96, 32.64, 30.70, 23.33. HRMS calcd for $C_{41}H_{35}N_6O$ $[M+H]^+$ 627.2867, found 627.2873.

4.4.20. N'-((9-((perfluorophenyl)methyl)-9H-pyrido[3,4-b]indol-1-yl)methylene)-1-methyl-9-benzyl-9H-pyrido[3,4-b]indole-3-carbohydrazide (**10t**)

A off white solid, yield: 92.3%, m.p. > 280℃. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.40 (s, 1H, *NH*), 8.91 (s, 1H, ArH), 8.76 (s, 1H, ArH), 8.55 (d, J = 5.2 Hz, 1H, ArH), 8.23 (d, J = 7.6 Hz, 1H, ArH), 8.13 (d, J = 8.0 Hz, 1H, ArH), 8.01 (d, J = 4.8 Hz, 1H, *CH* = N), 7.63–7.55 (m, 3H, ArH), 7.42–7.32 (m, 3H, ArH), 7.30–7.27 (m, 2H, ArH), 6.99–6.96 (m, 2H, ArH), 6.89 (s, 2H, ArCH_2), 5.83 (s, 2H, ArCH_2), 2.93 (s, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.54, 146.65 (m), 144.10 (m), 142.31, 142.18, 140.44, 138.63 (m), 138.44, 137.82, 137.76, 137.38, 137.20, 135.37, 132.34, 129.79, 129.37, 129.16, 129.12, 129.07, 127.79, 125.35, 125.30, 121.94, 121.74, 121.67, 121.55, 121.38, 121.15, 121.13, 115.43, 114.38, 113.87, 111.43 (m), 110.33, 110.20, 48.36, 39.11, 23.31. RMS calcd for $C_{39}H_{26}F_5N_6O$ $[M+H]^+$ 689.2083, found 689.2079.

4.5. MTT assay

Target compounds were assayed by the MTT method for determining cytotoxic activity as described previously [34]. The panel of cell lines included the human umbilical vein cell line EA.HY926, Lewis lung carcinoma (LLC), gastric carcinoma (BGC-823), murine colon carcinoma (CT-26), liver carcinoma (Bel-7402), and breast carcinoma (MCF-7). Cell lines were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. Growth inhibition rates were calculated with the following equitation: Inhibition ratio (%) = $\frac{OD_{compd}-OD_{blank}}{OD_{DMSO}-OD_{blank}}\times 100\%$. The half-maximal inhibitory concentration (IC_{50}) of each compound was calculated using GraphPad Prism software (version 6.0).

4.6. Evaluation of the antitumor activity of heterobivalent β-carbolines in mice

Sarcoma 180 and Lewis lung cancer cells were provided by the Shanghai Institute of Pharmaceutical Industry. Mice received subcutaneous injections of viable tumor cells (2×10^6 cells/mouse) in the armpit. At 7 days after mice were inoculated with these cells, the tumors were removed, and the cells were harvested. Each test compound was administered at a dosage of about one-fifth of the LD_{50} via i.p. injection to groups containing 9 female mice. Dosing was performed at 24 h after the inoculation and once a day for seven consecutive days. For most of the compounds, the amount was the maximum tolerated dose based on our preliminary studies. CTX (30 mg kg^{-1}) was used as the positive control and the vehicle as the negative control. The weights of the animals were recorded every three days. All of the animals were killed on the 21st day after tumor inoculation, and the tumors were excised and weighed. Tumor inhibition was calculated as follows:

$$(C - T)/C \times 100,$$

where T is the average tumor weight of the treated group and C is the average tumor weight of the negative control group.

4.7. Wound healing assay in vitro

HT-29 and LLC cells (about 1×10^6/mL) were seeded in a 24-well plate at a density that after 24 h of growth, and they were allowed to reach 90% confluence in complete medium. A single scratch wound was

created on the confluent monolayers using a micropipette tip across the center of the well and a straight line was scratched in one direction. Then, wounded monolayers were washed with phosphate buffer saline (PBS) to remove the detached cells, and each assay was carried out three times. After washing, fresh media with FBS was added, various concentrations of **10e** were added to their respective wells, and then they were incubated for 24 h. The medium in each well was discarded and washed several times with PBS. Cells migrated to the wound surface and the average distance of migrating cells was determined under an inverted microscope at designated time points. Pictures of three different regions of each wound were taken. The experiment was performed three times.

4.8. CAM assay in vivo

To evaluate the antiangiogenic activity of the heterobivalent β-carbolines, CAM assays were performed as previously described [34]. In brief, five-day-old fertilized chicken eggs were purchased from a local hatchery and incubated at 37 °C in an incubator. After injection of 0.5 mL of saline, the eggs were incubated horizontally to allow the CAMs to detach from the shells, making chambers. Compound **10e** was prepared in gelatin sponge discs at concentrations of 0.5, 5.0, and 50 μmoles/disc. CA4P was used as a positive control drug. Discs containing the vehicle only (DMSO) were used as negative controls. A small window opening was made in the shell, and the discs were applied onto the CAM. The opening was covered with sterilized surgical tape, and the embryos were incubated for 48 h at 38.5 °C. The CAMs were photographed under a dissecting microscope, and blood vessels in each CAM were counted. The results are presented as mean percentages of inhibition compared to the control ± SD, $n = 3$.

Acknowledgements

This research was supported by grants from the Scientific Research Innovation Project in Shihezi University (No. SHYL-YB201804), the Xinjiang Science and Technology Major Project (No. 2016A03005-1), the Program for Changjiang Scholars and Innovative Research Team in University (No. IRT15R46), and Xinjiang Huashidan Pharmaceutical Research Co. Ltd.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2020.103612.

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