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Original article

Synthesis and preliminary biological evaluation of novel taspine derivatives as anticancer agents

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ABSTRACT

Antiangiogenic therapy might represent a new promising anticancer therapeutic strategy. Taspine can significantly inhibit cell proliferation of human umbilical vein endothelial cells (HUVECs) induced by vascular endothelial growth factor-165, which is crucial for angiogenesis. In this study, a series of novel taspine derivatives were synthesized and screened for *in vitro* anticancer and antiangiogenesis activities. The majority of the derivatives demonstrated a moderate degree of cytotoxicity against human cancer cell lines. One of them (**14**) exhibited much better antiproliferative activity against CACO-2 ($IC_{50} = 52.5$ μM) and ECV304 ($IC_{50} = 2.67$ μM) cells than taspine did. Some of them were also effective in antiproliferative assays against HUVECs. The *in silico* estimate of solubility of title compounds were higher than that of taspine.

1. Introduction

Angiogenesis is a normal process in growth and development of blood vessels as well as wound healing [1]. Tumors follow a similar procedure to improve their own blood supply to ensure their aberrant growth [2]. Angiogenesis is a key event of tumor progression and metastasis and hence a target for cancer chemotherapy. Angiogenesis is primarily a receptor-mediated process that is mediated by growth factors that cause signal transduction via receptor tyrosine kinase (RTK). Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) have both been implicated in tumor angiogenesis. They are potent angiogenesis inducers *in vivo* and *in vitro*. Inhibition of the VEGF and bFGF RTKs has provided a new paradigm in the treatment of tumors. A great advantage of antiangiogenic agents is that they are likely to be much less toxic than the existing chemotherapy agents. Design and synthesis of novel small-molecule antiangiogenic agents are underway in modern medicinal chemistry.

Taspine was isolated from *Radix et Rhizoma Leonticis* (Hong Mao Qi in Chinese) by Li and He [3]. It has many pharmacological activities such as bacteriostatic, antibiotic, antiviral, anti-inflammatory, antiulcer, and cytotoxic activity, and can be used in wound healing [4]. Its anticancer and antiangiogenic properties have been demonstrated and it could be an ideal candidate for a chemotherapeutic agent [5]. Taspine can inhibit expression of VEGF in human umbilical vein endothelial cells (HUVECs), as well as proliferation of HUVECs induced by VEGF-165. In a further study, He et al. demonstrated that taspine could inhibit tumor angiogenesis, and one of its mechanisms may be that it inhibits VEGF receptor-2 (VEGFR-2) [6]. However, some studies have demonstrated that the use of taspine is limited by its low potency and poor solubility [7]. Poor solubility is a major problem in the biological testing of compounds, which has made formulating the compounds a challenge for *in vivo* studies of efficacy and pharmacokinetics [8]. This notwithstanding, taspine remains a good lead compound for the design of derivatives with increased activity and solubility. Taspine is a rigid tetracyclic molecule with a dilactonic, tertiary amine structure (Fig. 1) [9]. To research the individual contribution of the lactone ring and biphenyl scaffold of taspine to its biological activity, we designed and synthesized a series of novel ring-opened target compounds.

Alignment of multiple ligands based on shared pharmacophoric and pharmacosteric features is a long-recognized challenge in drug discovery and development. In this study, we applied pharmacophore modeling using GALAHAD (a genetic algorithm with linear assignment for hypermolecular alignment of datasets) to obtain more reasonable alignment results [10]. First, we selected 15 molecules with higher anticancer activities as a training set to

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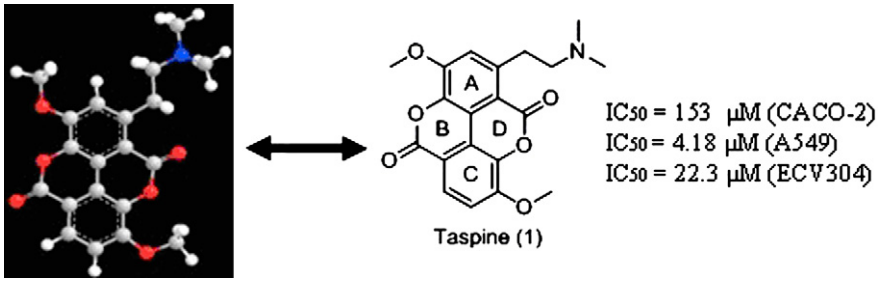


Fig. 1. The three-dimensional (3D) structure and antiproliferative activity of Taspine.

generate a pharmacophore model for molecular alignment. Then, a four-point pharmacophore model was generated from the 15 selected compounds. The pharmacophore features and their space locations are shown in Fig. 2. The hypothesis contained seven pharmacophoric features: four hydrophobic centers (cyan spheres), two hydrogen-bond donors (green), and one hydrogen-bond acceptor (purple). However, the title compounds did not fit the hypothesis perfectly with a maximum fit value of 4.

Our intention was to explore ring-opened derivatives, to see if they possess increased activity and solubility. We designed and synthesized four novel ring-opened target compounds (**13**–**16**) (Fig. 3). We envisaged breaking the diphenyl C–C bond and lactone C–O bond to yield four novel taspine derivatives. The preliminary biological test demonstrated that the ester bond of the B-ring was more important than that of D-ring in maintaining the biological activity of these compounds.

Biphenyl compounds are known for their varied biological activities, such as antibacterial, anti-inflammatory and anticancer activities. Our group has investigated biphenyl compounds and RTK inhibitors for several years. As a follow-up, a number of biphenyl derivatives were synthesized and evaluated as anticancer agents (**24**–**29**) (Fig. 3). The presence of chlorine and fluorine in a molecule enhances drug persistence and lipid solubility. Recently, we have synthesized compounds with a water-soluble secondary amine (dimethylamino, morpholine, and piperizine) and 3-chloro-4-flouroaniline linked to the positions 6- and 6′- of the biphenyl scaffold. The incorporation of second amines into the side chains of the molecules has increased the solubility of the compounds while retaining their biological activity. The second amine also mimics the *N,N*-dimethylaminoethyl side chain of taspine. In this paper, we described the synthesis and biological assay of taspine derivatives to develop novel and active anticancer agents.

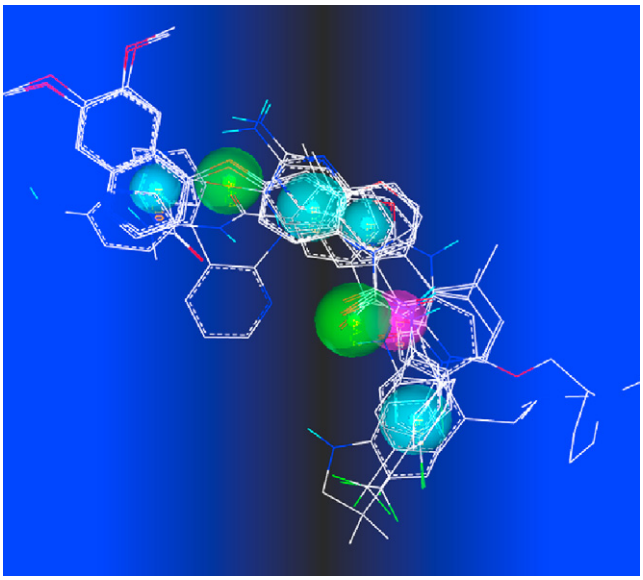


Fig. 2. Pharmacophore-based molecular alignment by using GALAHAD.

2. Chemistry

Four ring-opened title compounds were synthesized by the route outlined in Scheme 1. We used commercially available isovanillin as the starting material. At first, isovanillin was oxidized to give isovanillic acid with fused sodium hydroxide and potassium hydroxide. The methyl ester was prepared to avoid side-reactions of the carboxylate group. Refluxing of phenolic hydroxyl group with prenyl bromide in the presence of K_2CO_3 in anhydrous acetone yielded compound (**2**) [11]. A solution of (**2**) in *N,N*-dimethylaniline was heated to reflux for 8 h to give compound (**3**) [12]. The prenyl group was moved into the *para*-position of hydroxyl in this Claisen rearrangement process. The next step was the coupling of (**3**) to the carboxyl group of (**4**) by an ester bond with DCC and DMAP [13]. The oxidation of (**5**) with OsO_4/$NaIO_4$ produced aldehyde (**6**) [14], which reacted with dimethylamine, followed by reduction with $NaBH(OAc)_3$ to give (**7**) [15]. At last, benzyl deprotection of (**7**) with palladium/carbon in methanol gave the target compound (**13**) [16]. The other target compounds (**14**), (**15**) and (**16**) were synthesized in the same way from isovanillin.

Six biphenyl derivatives were prepared by the general routes described in Scheme 2. We also used isovanillin (**17**) as the starting material. Isovanillin (**17**) was converted into 2-bromoisovanillin (**18**) by reaction with bromine [17]. After protection of (**18**) as benzyl ether [18], the aldehyde group in (**19**) was oxidized to the carboxyl group to give (**20**) [19]. Amidation of (**20**) with methylamine yielded the monocyclic precursor (**22**) for the Ullmann reaction [20]. Biphenyl dimethane amide (**23**) was prepared by dimerization of (**22**) by employing a classical symmetrical Ullmann reaction [21]. Compound (**24**) could be readily obtained by deprotection of benzamide (**23**) under catalysis with palladium/carbon [22]. One or two of the hydroxyl groups in (**24**) was etherified with various alkyl halides in anhydrous acetone in the presence of K_2CO_3 to give (**25**) or (**26**) in different yields [23]. Finally, etherification of compound (**25**) yielded the target compound (**27**), (**28**) or (**29**).

3. Results and discussion

It is not always practical to perform experimental measurements, therefore, it is useful to predict molecular physicochemical properties rapidly such as Log P, Log D, pK_a, PSA, and solubility values [24]. Before biological evaluation, we evaluated a broad range of molecular physicochemical properties which had an impact on drug-like ADME parameters based on chemical structures (Table 1). From Table 1, we can see that solubility values of

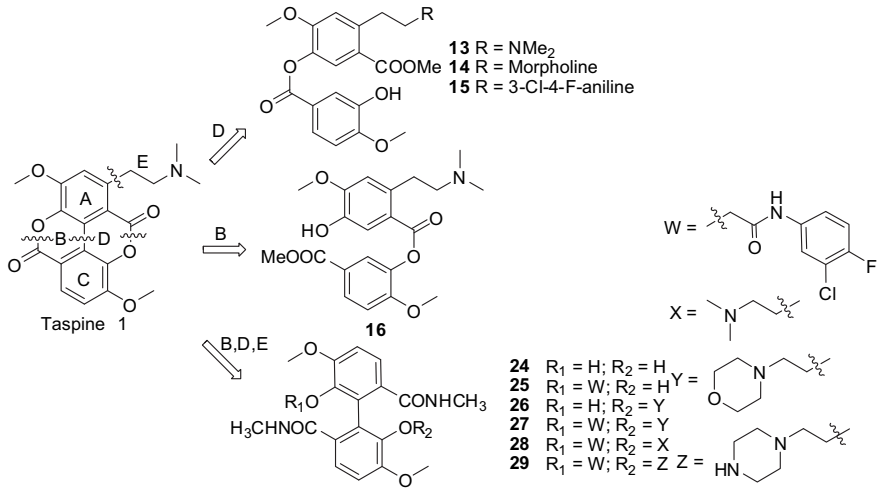
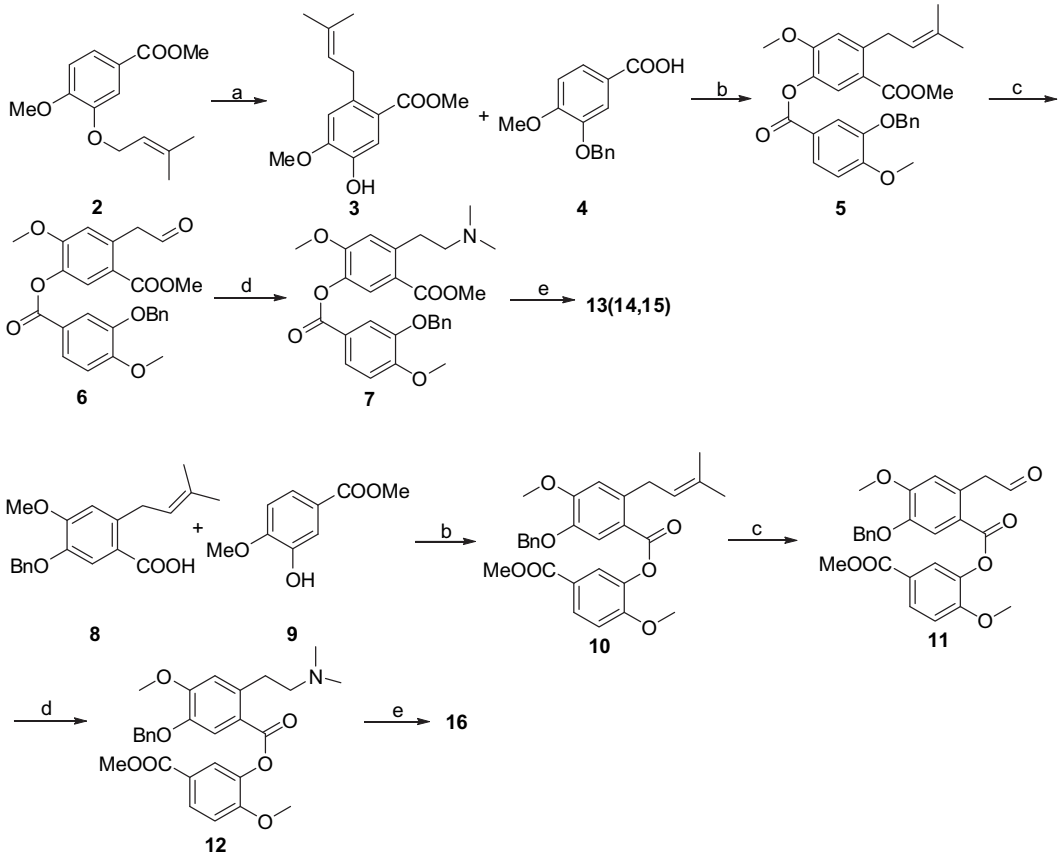


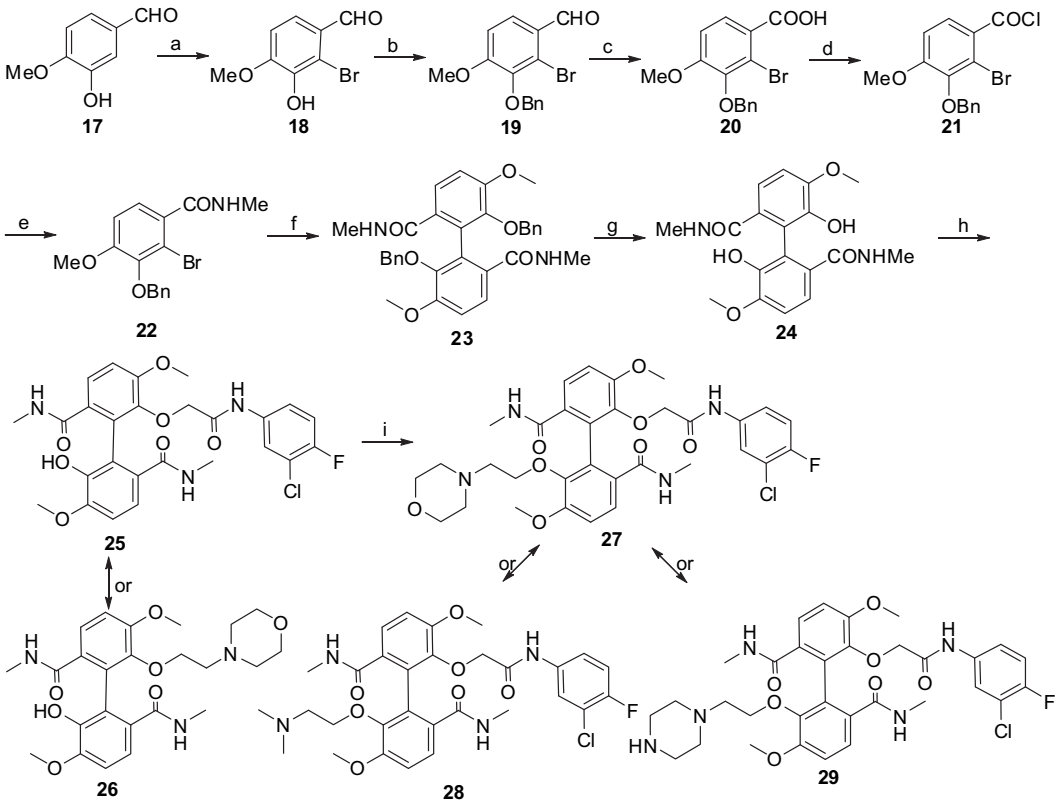
Fig. 3. Design of ring-opened taspine derivatives.

target compounds were higher than those of taspine. Although it was known that *in silico* estimates of pK_a and solubility were not exact, the calculated values suggested that there might be an improvement in physicochemical properties.

In vitro evaluation of cytotoxicity and antiproliferative activity of the synthesized compounds was carried out against various cancer and normal cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with taspine or gefitinib as a control (Table 2). The antiangiogenesis screening focused on the ability of the compounds to inhibit HUVEC growth *in vitro*. To search for novel small angiogenesis inhibitors, we also tested their antiproliferative activity against ECV304 human transformed endothelial cells. As shown in Table 2, most of the title compounds exhibit good growth inhibition of ECV304 proliferation in a dose-dependent manner, with IC_{50} values between 2.67 μM and 1717.70 μM.



Scheme 1. Preparation of target compounds (**13**–**16**). Reagents and Conditions: (a) *N,N*-dimethylaniline, N_2, reflux, 71%; (b) anhydrous THF, DCC, DMAP, (**5,** 71%; **10,** 78%); (c) OsO_4, $NaIO_4$, acetone/H_2O/t-BuOH, (**6**, 32%; **11**, 34%); (d) dimethylamine/Morpholine/3-Cl-4-F-aniline, THF, CH_2Cl_2, $NaBH(OAc)_3$, 25–40%; (e) H_2, Pd/C, 92–97%.



Scheme 2. Preparation of target compounds (**24**–**29**). Reagents and Conditions: (a) Fe, NaOAc, AcOH, Br_2, 81%; (b) BnCl, K_2CO_3, 95%; (c) NaH_2PO_4, $NaClO_2$, 30% H_2O_2, 93%; (d) $SOCl_2$, DMF(cat), CH_2Cl_2, 96%; (e) CH_2Cl_2, 30% CH_3NH_2, 85%; (f) Cu, DMF, 72%; (g) H_2, Pd/C; 97%; (h) K_2CO_3, DMF, (**26**, 64%; **25**, 76%); (i) K_2CO_3, EtOH, (**27**, 72%; **28**, 59%; **29**, 54%).

The activity of (**14**) was much better than that of taspine against CACO-2 (threefold) and ECV304 (about 10-fold) cells, with IC_{50} values of 52.5 μM and 2.67 μM, respectively. Compound (**14**) appeared to inhibit proliferation more potently in ECV304 cells, and this may be an indication that the compound was acting through inhibition of VEGFR-2. If the antiangiogenic properties of (**14**) were confirmed, the compound could represent a novel hit for lead optimization efforts. The biphenyl derivative (**25**) exhibited potent inhibitory activities against CACO-2 ($IC_{50} = 20.33$ μM), A549 ($IC_{50} = 73.51$ μM) and ECV304 ($IC_{50} = 72.17$ μM) cells. Among the biphenyl derivatives, compound (**28**) exhibited the best antiproliferative activity against ECV304 cells, with an IC_{50} value of 57.20 μM. The results indicated that the taspine derivatives could be served as antiangiogenic agents in cancer therapy.

We also evaluated the antiproliferative activity of biphenyl derivatives against several other human cancer cell lines using the MTT assay. The antiproliferative activity of biphenyl derivatives was tested against six different human cancer cell lines (Table 3). The tested compounds showed a broad spectrum of growth inhibition activity. Preliminary results showed that six biphenyl derivatives displayed potent antiproliferative activity, with IC_{50} values from 25.06 to 414.59 μM. Compound (**27**) exhibited the best activity against A375 ($IC_{50} = 25.06$ μM), MCF-7 ($IC_{50} = 60.38$ μM), HT-29 ($IC_{50} = 44.71$ μM) and PANC-1 ($IC_{50} = 110.43$ μM) cells. The presence of morpholine might be important for this activity. Replacement of morpholine with dimethylamine or piperizine led to a slight decrease in activity. Compound (**26**) was the most active compound against 7721, with an IC_{50} value of 78.16 μM, whereas compound (**25**) showed the best antiproliferative activity against HeLa ($IC_{50} = 53.37$ μM) cells.

Among the two series of compounds, (1**4**) and (**28**) exhibited the highest antiproliferative activity against ECV304 cells, which might

Table 1
Prediction of physicochemical properties of compounds.

	CLog P	Log D (PH = 7.4)	pK_a	Solubility (g/L) (PH = 7.4)	PSA[a]
Taspine	1.93	2.92	8.21	0.021	74.3
13	3.18	1.72	9.73; 8.75	0.14	94.53
14	3.33	2.65	9.02; 7.28	0.27	103.76
15	6.06	6.56	9.01; 3.65	0.061	103.32
16	3.18	1.97	9.55; 8.57	0.13	94.53
24	1.48	1.54	9.24; 8.31	36.57	117.12
25	0.50	0.47	8.52	0.61	135.22
26	1.96	2.08	8.57; 6.76	22.56	118.59
27	0.53	−0.71	8.62	2.21	127.46
28	0.03	−0.061	6.76	0.21	136.69
29	0.59	−1.28	9.29; 3.18	6.95	139.49

[a] PSA = Polar Surface Area ($Å^2$).

Table 2
Antiproliferative activities of target compounds on human cancer and normal cells.

Compound	Cell lines (IC_{50}, μM)				
	CACO-2[a]	A549[b]	ECV304[c]	EGFR cell[d]	A431[e]
Taspine	153	4.18	22.3	15.95	11.20
13	110.03	453	ND	ND	ND
14	52.5	158	2.67	ND	ND
15	ND	180	61	ND	ND
16	3100	111	ND	ND	ND
24	104.18	64.65	112.57	88.04	104.18
25	20.33	73.51	72.17	ND	ND
26	75.85	259.49	1364.84	116.02	75.85
27	3314.11	194.39	ND	ND	ND
28	167.18	240.51	57.20	ND	ND

ND, not determined.
[a] CACO-2, human epithelial colorectal adenocarcinoma cells.
[b] A549, carcinomic human alveolar basal epithelial cells.
[c] ECV304, human umbilical endothelial cell line.
[d] EGFR cell, Overexpressing Epidermal Growth Factor Receptor Stably cell (HEK293/EGFR).
[e] A431, model cell line express abnormally high levels of the EGFR.

Table 3
Antiproliferative activities of biphenyl derivatives on human cancer cells.

Compound	Cell lines (IC_{50}, μM)					
	A375[a]	7721[b]	Hela[c]	MCF-7[d]	HT-29[e]	PANC-1[f]
Gefitinib	20.55	33.12	45.59	69.16	16.93	69.32
24	105.59	95.69	97.61	ND	200.41	217.09
25	217.42	109.44	53.37	109.16	213.70	191.16
26	174.07	78.16	414.59	83.68	122.11	165.59
27	25.06	194.09	100.26	60.38	44.71	110.43
28	242.11	124.19	116.68	123.02	133.48	159.09
29	ND	125.53	ND	ND	47.17	ND

ND, not determined.
[a] A375, human amelanotic melanoma cells.
[b] 7721, Human hepatoma cell line.
[c] Hela, an immortal cell line.
[d] MCF-7, human breast cancer cell line.
[e] HT-29, human colon adenocarcinoma grade II cell line.
[f] PANC-1, human pancreatic carcinoma, epithelial-like cell line.

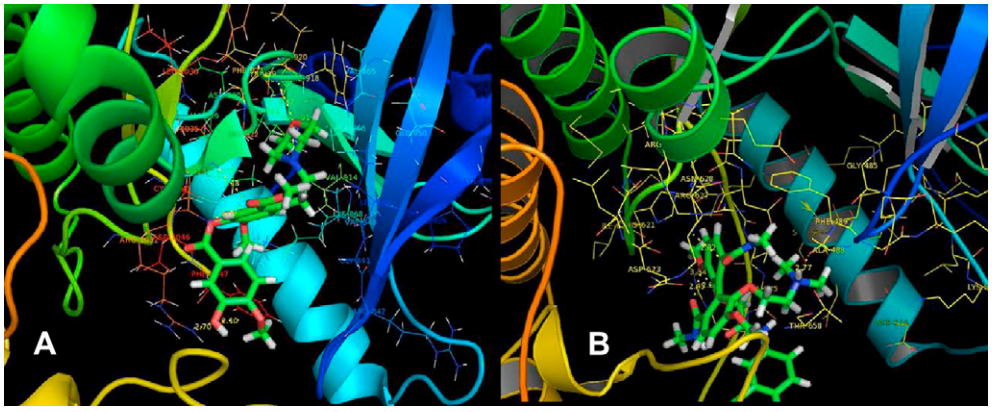


Fig. 4. (A) The compound **14** was built and docked into the active site of VEGFR-2 (PDB ID: 3C7Q). (B) The compound **28** was built and docked into the active site of bFGFR-1 (PDB ID: 3C4F). The docking result was showed by PyMOL.

have been related to RTK inhibition. It is known that antiangiogenic agents also manifest significant inhibitory activity against the receptors for VEGF and bFGF. To investigate the antiangiogenesis mechanism of taspine derivatives, a molecular docking study of target compounds with VEGFR-2 and bFGFR-1 was performed using SYBYL 7.0 to identify their binding mode with the enzyme. Compounds (**14**) and (**28**) were docked into the potential binding sites of VEGFR-2 and bFGFR-1, respectively (Fig. 4). The docking results indicated that the binding energies of the two compounds with VEGFR-2 and bFGFR-1 were both lower than that of taspine. The two compounds both bound to the ATP-binding site in the cleft between the N- and C-terminal lobes of the kinase domain. The binding hypothesis could provide valuable information for structure-based design of taspine analogs.

4. Conclusions

In the present study, 10 novel ring-opened taspine derivatives were described. All the compounds were tested and most of them showed moderate to good anticancer activity. Compound (**1**4), methyl 5-[(3-hydroxy-4-methoxybenzoyl) oxy]-4-methoxy-2-(2-morpholin-4-ylethyl)benzoate, showed the highest antiproliferation activity in CACO-2 and ECV304 cell lines. Some of the compounds showed good antiproliferative activity against colon (HT29), breast (MCF-7), lung (A549), rectum (CACO-2), skin (A375), hepatoma (7721), and pancreatic (PANC-1) cancers cell lines.

In summary, the novel taspine derivatives were designed and synthesized. Preliminary bioassays indicated that the majority of the derivatives demonstrated moderate antiangiogenic and anticancer activities. We demonstrated that the lactone ring B is important for activity, while the lactone ring D can be opened, thus retaining and even improving the antiproliferative properties of taspine. The docking results and the antiproliferative activity against HUVECs suggest that these taspine derivatives are potent RTK inhibitors. The data indicate that these compounds could also exhibit an antiangiogenic mechanism of action, similar to that of taspine, and could therefore be a promising starting point for further medicinal chemistry efforts. Our attempts to optimize the physicochemical and biological properties of taspine analogs will be reported in due course.

5. Experimental

5.1. Antiproliferative activity of taspine derivatives

Growth inhibitory activities were evaluated on the following cell lines: CACO-2, A549, ECV304, EGFR cell (cell of overexpressing Epidermal Growth Factor Receptor Stably HEK293/EGFR), A431, A375, 7721, Hela, MCF-7, HT-29, and PANC-1. The effects of the compounds on cell viability were evaluated using the MTT assay [23]. Exponentially growing cells were harvested and plated in 96-well plates at a concentration of 1×10^4 cells/well, and incubated for 24 h at 37 °C. The cells in the wells were respectively treated with target compounds at various concentrations for 48 h. Then, 20 μL MTT (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. After the supernatant was discarded, 150 μL DMSO was added to each well, and the absorbance values were determined by a microplate reader (Bio-Rad Instruments) at 490 nm.

5.2. Chemistry: general procedures

All reactions except those in aqueous media were carried out by standard techniques for the exclusion of moisture. Solvents were purified before use according to standard procedures. All reactions were monitored by thin layer chromatography on 0.25-mm silica gel plates (60GF-254) and visualized with UV light. All melting points were determined on a Beijing micro-melting point apparatus and were uncorrected. ^{1}H-NMR spectra were recorded on a Bruker AVANCF300 MHZ instrument in $CDCl_3$ solution with TMS a**s** internal standard. Mass spectra were performed on a Shimadzu GC-MS-QP2010 instrument.

5.2.1. Methyl 5-hydroxy-4-methoxy-2-(3-methylbut-2-enyl) benzoate (**3**)

A solution of (**2**) (4.06 g, 16.24 mmol) in *N,N*-dimethylaniline (20 mL) was refluxed under nitrogen for 8 h, then cooled to room temperature, added ethyl acetate (150 mL). The organic layer was washed with 2 N HCl (5 × 30 mL), saturated $NaHCO_3$ (2 × 30 mL) and saturated NaCl (2 × 30 mL), dried over Na_2SO_4. The solvent was filtered and concentrated to give pale yellow oil, which was purified by flash chromatography on silica gel to give (**3**) (2.88 g, 71.0%) as a white solid, mp 76.0–77.0 °C. EI-MS(m/z): 250.0 (M^+), ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.71 (s, 6H), 3.68 (d, 2H, $J = 6.0$ Hz), 3.90 (s, 3H), 4.07 (s, 3H), 5.27 (t, 1H, $J = 6.6$ Hz), 5.50 (s, 1H), 6.69 (s, 1H), 7.51 (s, 1H).

5.2.2. Methyl 5-(3-(benzyloxy)-4-methoxybenzoyloxy)-4-methoxy-2-(3-methylbut-2- enyl)benzoate (**5**)

A solution of DCC (*N,N′*-dicyclohexylcarbodiimide) (0.99 g, 4.8 mmol) in 10 mL of anhydrous THF was added dropwise to a mixture of compound (**4**) (1.23 g, 4.8 mmol), DMAP (4-(dimethylamino)pyridine) (0.05 g, 0.4 mmol) and (**3**) (1.0 g, 4.0 mmol) in 30 mL of anhydrous THF at 0 °C. The reaction mixture was stirred at room temperature for 48 h. The precipitated DCU (*N,N′*-dicyclohexylurea) was filtered off and THF was evaporated in cacuo. The residues obtained after evaporation were dissolved in 100 mL ethyl acetate. The organic layer was washed with 2 N HCl (3 × 30 mL), saturated $NaHCO_3$ (3 × 30 mL) and saturated NaCl (3 × 30 mL), dried over Na_2SO_4. The solvent was evaporated to give a residue which was chromatographed on a silica column to give (**5**) (1.53 g, 78.0%) as white solid, mp 115.0–116.0 °C. EI-MS(m/z): 489.9 (M^+), ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 1.67 (s, 6H), 3.76 (s, 3H), 3.77 (s, 3H), 3.71 (d, 2H, $J = 6.0$ Hz), 3.89 (s, 3H), 5.13 (s, 2H), 5.23 (t, $J = 6.0$ Hz, 1H), 6.79 (s, 1H), 6.89 (d, $J = 8.5$ Hz, 1H), 7.22–7.33 (m, 5H), 7.39 (s, 1H), 7.69 (s, 1H), 7.79 (d, $J = 8.5$ Hz, 1 H).

5.2.3. Methyl 5-(3-(benzyloxy)-4-methoxybenzoyloxy)-4-methoxy-2-(2-oxoethyl) benzoate (**6**)

OsO_4 (23.4 mg, 5% mol) was added to a stirred solution of (**5**) (0.90 g, 1.84 mmol) in 3:1:1 acetone-*t*-BuOH–H_2O (40 mL) (the starting material was dissolved in acetone-*t*-BuOH, and H_2O was added last). After stirred keeping from light at room temperature for 30 min, $NaIO_4$ (51.18 g, 5.52 mmol) was added in one portion. Stirring was continued for 12 h. The reaction miture was filtered through diatomite, washed with acetone. The filtrate was concentrated in vacuum under 20 °C, and the residues were dissolved in 150 mL ethyl acetate. The organic layer was washed with 5% aquous $NaHSO_3$ (3 × 20 mL) and water (3 × 30 mL), dried over Na_2SO_4 and evaporated. Flash chromatography of the residue over silica gel, using 1:1-EtOAc-petroleum ether, gave aldehyde (**6**) (0.27 g, 32%) as white solid, 169.5–170.5 °C, EI-MS(m/z): 463.9 (M^+), ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 3.85 (s, 3H), 3.88 (s, 3 H), 3.99 (s, 3H), 4.25 (s, 2H), 5.17 (s, 2H), 6.97 (d, $J = 6.0$ Hz, 1H), 7.28–7.37 (m, 5H), 7.44 (s, 1H), 7.69 (s, 1H), 7.72(m, 1H), 7.91 (s, 1H), 9.85 (s, 1H).

5.2.4. Methyl 5-(3-(benzyloxy)-4-methoxybenzoyloxy)-2-(2-(dimethylamino)ethyl)-4- methoxybenzoate (**7**)

Aldehydr (**6**) (0.10 g, 0.22 mmol) was dissolved in CH_2Cl_2 (20 mL) and methol (10 mL) under a nitrogen atmosphere at room temperature, and dimethylamine (0.44 mmol) in THF was added. After stirring for 4 h, $NaBH(OAc)_3$ (0.058 g, 0.26 mmol) was gradually added over 10 min to the reaction mixture. The mixture was stirred for another 12 h at room temperature. The solvent was evaporated in vacuuo and the residue was purified by column chromatography to give (**7**) (0.042 g, 38.0%) as a white solid, mp 147.5–148.5 °C, EI-MS(m/z): 493.1 (M^+), ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 2.55 (s, 6H), 2.68 (t, $J = 7.6$ Hz, 2H), 3.59 (t, $J = 7.9$ Hz, 2H), 3.85 (s, 3H), 3.87 (s, 3 H), 4.01 (s, 3H), 5.20 (s, 2H), 6.96 (s, 1H), 7.26 (s, 1H), 7.35–7.47 (m, 5H), 7.73–7.79 (m, 2H), 7.85(s, 1H).

5.2.5. Methyl 2-(2-(dimethylamino)ethyl)-5-(3-hydroxy-4-methoxybenzoyloxy)-4- methoxybenzoate (**13**)

Compound (**7**) (0.16 g, 0.32 mmol) and Pd/C (0.02 g) in methanol (25 mL) were stirred at room temperature under H_2 atmosphere for 48 h. After completion of the reaction, the solid materials

were separated by filtration over Celite followed by washed with methanol. After removal of the solvent, the crude product was purified by column chromatography to give (**13**) (0.12 g, 97.0%) as a white solid, mp 112.5–114.5 °C, EI-MS(*m*/*z*): 403.1 (M^+), ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 2.52(s, 6H), 2.71 (t, $J = 7.3$ Hz, 2H), 3.62 (t, $J = 7.0$ Hz, 2H), 3.84 (s, 3H), 3.89 (s, 3 H), 3.98 (s, 3H), 6.89 (s, 1H), 7.08 (d, $J = 8.7$ Hz, 1H), 7.54 (d, $J = 9.0$ Hz, 1H), 7.67 (s, 1H), 7.81 (s, 1H).

Compounds (**14**), (**15**) and (**16**) were prepared by using the general procedure described above.

*5.2.5.1. Methyl-5-[(3-hydroxy-4-methoxybenzoyl)oxy]-4-methoxy-2-(2-morpholin-4-ylethyl)benzoate (**14**).* mp 121.0–124.5 °C, EI-MS (*m*/*z*): 445.2 (M^+), ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 2.55–2.63 (m, 4H), 2.82 (t, $J = 8.0$ Hz, 2H), 3.42–3.49 (m, 4H), 3.71 (t, $J = 7.8$ Hz, 2H), 3.82 (s, 3H), 3.90 (s, 3 H), 3.97 (s, 3H), 6.76 (s, 1H), 7.00 (d, $J = 9.0$ Hz, 1H), 7.42 (d, $J = 8.7$ Hz, 1H), 7.73 (s, 1H), 7.87 (s, 1H).

5.2.5.2. Methyl-2-{2-[(3-chloro-4-fluorophenyl)amino]ethyl}-5-[(3-hydroxy-4-methoxybenzoyl) oxy]-4-methoxybenzoate (15). mp 101.0–103.5 °C, EI-MS(*m*/*z*): 503.7 (M^+), ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 2.49 (t, $J = 7.5$ Hz, 2H), 3.52 (t, $J = 7.2$ Hz, 2H), 3.81 (s, 3H), 3.87 (s, 3 H), 3.95 (s, 3H), 6.88 (d, $J = 7.5$ Hz, 1H), 6.94 (s, 1H), 6.97–7.10 (m, 2H), 7.19–7.21 (m, 1H), 7.54 (d, $J = 9.0$ Hz, 1H), 7.68 (s, 1H), 7.77 (s, 1H).

*5.2.5.3. 2-Methoxy-5-(methoxycarbonyl)phenyl-2-[2-(dimethylamino)ethyl]-5-hydroxy-4-methoxybenzoate (**16**).* mp 107.0–109.5 °C, EI-MS(*m*/*z*): 402.9 (M^+), ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 2.62 (s, 6H), 3.55 (t, $J = 6.6$ Hz, 2H), 3.83 (s, 3H), 3.85 (s, 3 H), 3.98 (s, 3H), 4.01 (t, $J = 6.9$ Hz, 2H), 6.66 (s, 1H), 7.13 (d, $J = 9.0$ Hz, 1H), 7.72 (s, 1H), 7.86 (d, $J = 9.0$ Hz, 1H), 7.93 (s, 1H).

5.2.6. 2-Bromo-3-hydroxy-4-methoxybenzaldehyde (18)

To a mixture of (**17**) (20.0 g, 0.132 mol), NaOAc (21.59 g, 0.263 mol) and iron powder (0.68 g, 0.012 mol) was added glacial acetic acid (120 mL). The mixture was stirred at room temperature for 30 min. Br_2 (7.0 mL, 0.14 mol) in glacial acetic acid (30 mL) was added dropwise into the above mixture at 23–25 °C. The mixture was stirred at the same temperature for 3 h. Ice-water (250 mL) was added to the mixture and stirred for another 1 h and filtered. The solid obtained as dried and recrystallized from EtOH to give (**18**) (24.59 g, 81%) as a gray solid. mp 206–207 °C; EI-MS(*m*/*z*): 230.9 ($[M + H]^+$), ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 4.01 (s, 3H), 6.07 (s, 1H), 6.93 (d, $J = 8.5$ Hz, 1H), 7.58 (d, $J = 8.5$ Hz, 1H), 10.26 (s, 1H).

*5.2.7. 3-(Benzyloxy)-2-bromo-4-methoxybenzaldehyde (**19**)*

To a suspension of (**18**) (6.90 g, 30 mmol) in dehydrated alcohol (120 mL) was added anhydrous potassium carbonate (12.44 g, 90 mmol) and benzyl chloride (5.20 mL, 45 mmol). The mixture was refluxed for 4 h. Filtration and evaporation of alcohol was done in a vacuum. The residue was extrated with EtOAc (2 × 70 mL). The combined organic layers were washed with H_2O (3 × 40 mL), 2 M NaOH (3 × 40 mL), 2 M HCl (3 × 40 mL) and brine (2 × 40 mL), dried over Na_2SO_4, and concentrated to give (**19**) (9.12 g, 95%) as a yellow solid. mp 79–81 °C; EI-MS(*m*/*z*): 320.9 ($[M + H]^+$), ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 3.96 (s, 3H), 5.03 (s, 2H), 6.98 (d, $J = 9.2$ Hz, 1H), 7.35–7.54 (m, 5H), 7.76 (d, $J = 8.4$ Hz, 1H), 10.27 (s, 1H).

*5.2.8. 3-(Benzyloxy)-2-bromo-4-methoxybenzoic acid (**20**)*

To a solution of (**19**) (9.60 g, 30 mmol) in THF (100 mL) was added distilled H_2O (40 mL) and NaH_2PO_4 (2.16 g, 18 mmol). The mixture was stirred at room temperature for 10 min. $NaClO_2$ (8.96 g, 99 mmol) and 30% H_2O_2 (6.8 mL, 66 mmol) in distilled H_2O (25 mL) were added into the above mixture. The mixture was stirred at the same temperature for 3 h. THF was evaporated under vacuum and the residue was extrated with EtOAc (2 × 80 mL). The combined organic layers were washed with (3 × 30 mL) and the product was extracted with 2 M NaOH (5 × 30 mL). The aqueous phase was acidified with concentrated HCl and the solid obtained was collected by filtration and dried to give (**20**) (9.37 g, 93%) as a white solid. mp 159–161 °C; EI-MS(*m*/*z*): 336.9 ($[M + H]^+$), ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 3.94 (s, 3H), 5.03 (s, 2H), 6.93 (d, $J = 9.2$ Hz, 1H), 7.37–7.56 (m, 5H), 7.87 (d, $J = 9.1$ Hz, 1H).

*5.2.9. 3-(Benzyloxy)-2-bromo-4-methoxy-N-methylbenzamide (**22**)*

To a suspension of (**20**) (5.04 g, 15 mmol) in CH_2Cl_2 (50 mL) was added thionyl chloride (4.37 mL, 60 mmol) and a catalytic amount of DMF. The mixture was stirred at room temperature for 2 h. The solvent was removed by evaporation in vacuo to give 5.31 g (100%) of the acid chloride of (**20**).

To a solution of 30% aqueous methylamine (10.31 mL, 90 mmol) in THF (20 mL) was added a solution of the acid chloride (**21**) (5.31 g, 15 mmol) obtained above in CH_2Cl_2 (15 mL) dropwise with cooling by an ice-water bath. The mixture was stirred at room temperature for 2 h. The reaction mixture was diluted with AcOEt, washed with aqueous saturated $NaHCO_3$, water and brine, and dried over $NaSO_4$. Filtration and concentration in vacuo and purification by silica gel flash chromatography ($CHCl_3$/MeOH = 30:1) gave 4.46 g (85%) of (**22**) as a white solid. mp 142–144 °C; EI-MS(*m*/*z*): 350.9 ($[M + H]^+$), ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 3.00 (d, $J = 4.3$ Hz, 3H), 3.89 (s, 3H), 5.01 (s, 2H), 6.13 (br, 1H), 6.91 (d, $J = 7.2$ Hz, 1H), 7.37–7.56 (m, 5H), 7.55 (d, $J = 7.0$ Hz, 1H).

*5.2.10. 6,6′-Bis(benzyloxy)-5,5′-dimethoxy-N,N′-dimethylbiphenyl-2,2′- dicarboxamide(**23**)*

To a solution of (**22**) (3.49 g, 10 mmol) in anhydrous DMF (25 mL) was added freshly activated copper powder (6.4 g, 100 mmol) under nitrogen atmosphere, and the mixture was refluxed for 4 h at 150–160 °C. The mixture was filtered and DMF was evaporated under vacuum. The residue was extrated with $CHCl_3$ (3 × 50 mL), and the combined organic layers were washed with 2 M HCl (3 × 30 mL) and brine (2 × 30 mL), dried over Na_2SO_4, and concentrated. The residue was chromatographed on silica gel (PE/AcOEt = 1:1) to give (**23**) (1.95 g, 72%) as a white solid. mp 178–180 °C; EI-MS(*m*/*z*): 540.1 ($[M]^+$), ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 2.65 (d, $J = 4.6$ Hz, 6H), 3.87 (s, 6H), 4.73 (d, $J = 10.9$ Hz, 1H), 4.82 (d, $J = 10.8$ Hz, 1H), 6.94–7.19 (m, 12H), 7.33 (d, $J = 8.4$ Hz, 2H).

*5.2.11. 6,6′-Dihydroxy-5,5′-dimethoxy-N,N′-dimethylbiphenyl-2,2′-dicarboxamide (**24**)*

To a solution of (**23**) (3.11 g, 5.76 mmol) in anhydrous methanol (100 mL) was added 10% Pd/C (0.31 g) (10%) under hydrogen atmosphere. The mixture was stirred at room temperature until no starting material could be observed by TLC. Pd/C was filtered and washed with methanol (400 mL). Then the combined filtrates were evaporated under vacuum to give (**24**) (2.01 g, 97%) as a gray solid. mp 166–168 °C; EI-MS(*m*/*z*): 360.1 ($[M]^+$), ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 2.74 (d, $J = 4.2$ Hz, 6H), 3.92 (s, 6H), 6.89 (d, $J = 8.4$ Hz, 2H), 7.16 (d, $J = 8.5$ Hz, 2H).

*5.2.12. 6-Hydroxy-5,5′-dimethoxy-N,N′-dimethyl-6′-(2-morpholin-4 -ylethoxy)biphenyl-2,2′- dicarboxamide (**26**)*

A solution of (**24**) (3.13 g, 8.7 mmol) and potassium carbonate (2.41 g, 17.5 mmol) in DMF (50 mL) was stirred at room temperature for 30 min and then 4-(3-chloropropyl)morpholine was added. This mixture was stirred at 80 °C in a water bath for 10 h under nitrogen atmosphere. The reaction was poured on crushed ice-water mixture and extracted with ethyl acetate (4 × 50 mL).

Organic layers were combined, extracted with brine (3 × 30 mL), dried over Na_2SO_4 and solvent was removed. The crude material was crystallized from AcOEt/PE to afford (**26**) (2.64 g, 64%) as a white solid. mp 172–175 °C; EI-MS(*m*/*z*): 473.2 ($[M]^+$), ^1H NMR (300 MHz, $CDCl_3$) δ ppm 2.14–2.35 (m, 6H), 2.63 (d, $J = 4.2$ Hz, 3H), 2.76 (d, $J = 4.3$ Hz, 3H), 3.61–3.65 (m, 4H),3.86 (s, 3H), 3.92 (s, 3H), 4.11–4.15 (m, 2H), 6.84 (d, $J = 8.4$ Hz, 1H), 6.89 (d, $J = 8.5$ Hz, 1H), 7.14 (d, $J = 8.4$ Hz, 1H), 7.38 (d, $J = 8.8$ Hz, 1H).

Compound (**8**) was prepared in the same way.

*5.2.12.1. 6-{2-[(3-Chloro-4-fluorophenyl)amino]-2-oxoethoxy}-6′-hydroxy-5,5′-dimethoxy-N,N′- dimethylbiphenyl-2,2′-dicarboxamide (**25**).* Yield, 76%, white crystalline powder, mp 159–162 °C; EI-MS (*m*/*z*): 545.1 ($[M]^+$), ^1H NMR (300 MHz, $CDCl_3$) δ ppm 2.63 (s, 3H), 2.84 (s, 3H), 3.83 (s, 3H), 3.91 (s, 3H), 4.59 (s, 1H), 4.64(s, 1H), 5.72 (br, 1H), 6.33(br, 1H), 6.72 (d, $J = 7.8$ Hz, 1H), 6.95 (d, $J = 7.9$ Hz, 1H), 7.08 (d, $J = 8.7$ Hz, 1H), 7.26–7.29 (m, 3H), 8.00 (d, $J = 6.3$ Hz, 1H), 8.92 (s, 1H).

*5.2.13. 6-{2-[(3-Chloro-4-fluorophenyl)amino]-2-oxoethoxy}-5,5′-dimethoxy-N,N′-dimethyl-6′- (2-morpholin-4-ylethoxy)biphenyl-2,2′-dicarboxamide (**27**)*

To a suspension of (**25**) (0.30 g, 0.55 mmol) in dehydrated alcohol (50 mL) were added anhydrous potassium carbonate (0.46 g, 3.3 mmol) and *N*-(2-chloroethyl)morpholine hydrochloride (0.21 g, 1.1 mmol). The mixture was refluxed for 8 h. Filtration and evaporation of alcohol was done in a vacuum. The residue was extrated with EtOAc (2 × 30 mL). The combined organic layers were washed with H_2O (3 × 10 mL), 2 M NaOH (3 × 10 mL), 2 M HCl (3 × 10 mL) and brine (2 × 10 mL), dried over Na_2SO_4, and concentrated to give (**27**) (0.25 g, 72%) as a white crystalline powder, mp 165–167 °C; EI-MS(*m*/*z*): 658.1 ($[M]^+$), ^1H NMR (300 MHz, $CDCl_3$) δ ppm 2.18–2.33 (m, 6H), 2.62 (d, $J = 3.8$ Hz, 3H), 2.82 (d, $J = 3.7$ Hz, 3H), 3.62–3.67 (m, 4H), 3.80 (s, 3H), 3.89 (s, 3H), 3.97–4.02 (m, 2H), 4.69 (s, 1H), 4.73 (s, 1H), 6.79 (d, $J = 8.5$ Hz, 1H), 6.90 (d, $J = 8.2$ Hz, 1H), 7.11 (d, $J = 8.4$ Hz, 2H), 7.67–7.72 (m, 3H), 8.04 (d, $J = 8.2$ Hz, 2H).

Compound (10) was prepared in the same way.

*5.2.13.1. 6-{2-[(3-Chloro-4-fluorophenyl)amino]-2-oxoethoxy}-3,3′-dimetlhoxy-N,N′-dimethyl-6′- (2-morpholin-4-ylethoxy)biphenyl-2,2′-dicarboxamide (**28**).* Yield, 59%, mp 138–141 °C; EI-MS(*m*/*z*): 617.1 ($[M]^+$), ^1H NMR (300 MHz, $CDCl_3$) δ ppm 2.38 (s, 6H), 2.67 (d, $J = 4.2$ Hz, 3H), 2.72 (t, $J = 6.3$ Hz, 2H), 2.88(d, $J = 3.9$ Hz, 3H), 3.84 (s, 3H), 3.92 (s, 3H), 4.14 (t, $J = 6.0$ Hz, 2H), 4.72 (s, 1H), 4.75 (s, 1H), 6.84 (d, $J = 8.2$ Hz, 1H), 6.92 (d, $J = 8.1$ Hz, 1H), 7.13 (d, $J = 8.5$ Hz, 2H), 7.69–7.74 (m, 3H), 8.01 (d, $J = 8.3$ Hz, 2H).

*5.2.13.2. 6-{2-[(3-Chloro-4-fluorophenyl)amino]-2-oxoethoxy}-5,5′-dimethoxy-N,N′-dimethyl-6′-(2-piperazin-1-ylethoxy)biphenyl-2,2′-dicarboxamide (**29**).* Yield, 54%, mp 192–195 °C; EI-MS(*m*/*z*): 657.1 ($[M]^+$), ^1H NMR (300 MHz, $CDCl_3$) δ ppm 2.54–2.67 (m, 6H), 2.78 (s, 3H), 3.07(s, 3H), 3.42–3.57 (m, 4H), 3.78 (s, 3H), 3.91 (s, 3H), 4.57 (s, 1H), 4.62(s, 1H), 6.80 (d, $J = 8.3$ Hz, 1H), 6.98 (d, $J = 8.0$ Hz, 1H), 7.06 (d, $J = 8.8$ Hz, 2H), 7.43 (d, $J = 8.3$ Hz, 2H), 7.91–7.99 (m, 3H).

5.3. Molecular modeling

Molecular docking method was often applied to predict the binding conformation of ligand when there was no available cocrystal structure. Here molecule docking was performed using the Sybyl/FlexX (Tripos Inc.) based on the crystal structures of VEGFR-2 and bFGFR-1 taken from the Protein Data Bank. Hydrogens were added and minimized using the Tripos force field and Pullman charges. Model compounds were constructed with the Sybyl/Sketch module (Tripos Inc.) and optimized using Powell's method with the Tripos force field with convergence criterion set at 0.05 kcal/(Å mol), and assigned with the Gasteiger-Hűckel method [25]. The residues in a radius of 6.5 Å around BIBF 1120 (the ligand of VEGFR-2 in the crystal structure 3C7Q) in VEGFR-2, and C4F (the ligand of bFGFR-1 in the crystal structure 3C4F) in bFGFR-1 were selected as the active site. Other docking parameters implied in the program were kept at default. Virtual docking of VEGFR-2 and bFGFR-1 in complex with the inhibitors provided a basis for further studies aimed at identifying inhibitors of VEGF- and bFGF-induced angiogenesis, and valuable information for structure-based design of second generation inhibitors.

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