European Journal of Medicinal Chemistry 46 (2011) 3420–3427

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Semi-synthesis and antitumor activity of 6-isomers of 5, 8-*O*-dimethyl acylshikonin derivatives

Wen Zhou [a,1], Xu Zhang [a,1], Ling Xiao [b], Jing Ding [a], Quan-Hua Liu [b], Shao-Shun Li [a,*]

[a] School of Pharmacy, Shanghai Jiaotong University, 800 Dongchuan Road, Shanghai 200240, China
[b] Shanghai Institute of Pharmaceutical Industry, 1111 North Zhongshan Road, Shanghai 200437, China

ARTICLE INFO

Article history:
Received 6 February 2011
Received in revised form
2 May 2011
Accepted 4 May 2011
Available online 12 May 2011

Keywords:
Shikonin derivatives
Semi-synthesis
Antitumor activity
Positional isomer
Structure-activity relationship

ABSTRACT

We recently discovered that 5, 8-*O*-dimethyl acylshikonin derivatives displayed the selectivity towards MCF-7 and no toxicity to normal cells. Herein, a series of the corresponding 6-isomers of 5, 8-*O*-dimethyl acylshikonin derivatives were synthesized starting from shikonin. In vitro evidence of the cytotoxicities indicated that most of thecompounds were more active than or comparative to shikonin and retained the selectivity against MCF-7, MDA-MB-231 besides no toxicity in the normal cells. Also, in vivo anticancer activity of the positional isomers **5p, 6c** further showed that 6-isomers of 5, 8-*O*-dimethyl acylshikonin derivatives were more active than their corresponding 2-isomers. Thus, we may conclude that the position of the side chain of shikonin attached to 5,8-dimethoxy -1,4-naphthoquinone is associated with the antitumor activity.

1. Introduction

The pharmacophore of shikonin was found to be naphthazarin ring [1], which was probably complicated in forming covalent bond with cellular nucleophiles such as DNA, proteins etc and generating superoxide anions via the redox cycling to kill cells [2–4]. The permethylated products of naphthazarin and its derivatives, 5,8-dimethoxy-1,4- naphthoquinone (DMNQ) and its analogues, decreased slightly the cytotoxicity but appeared to increase the T/C values, implying that the disappearance of tautomerism of naphthazarin and the electron density increased in the quinonoid moiety of DMNQ will benefit antitumor activities in vivo [5]. In our continuing drug discovery program most of 5, 8-*O*- dimethyl acylshikonin derivatives, expressing higher selectivity to MCF-7 and no toxicity to the normal cell, exhibited a greater potency in the growth inhibition of subcutaneous S-180 carcinoma, as well as less toxicity in vivo than lead compound shikonin [6]. However, it is well-known that the permethylation of naphthazarin derivatives could form two positional isomers as 6-substituted DMNQ (6-isomer) and 2-substituted DMNQ (2-isomer) (Fig. 1), and shikonin is certainly no exception. As evidenced by Ahn and his team, compared with 2-isomers, 6-isomers demonstrated a higher potency in the inhibitory effects on DNA topoisomerase-I and the cytotoxicity against L1210 cell [7–9], to a large extent suggesting the importance of the position of side chain on quinonoid moiety in effecting the antitumor bioactivities. Therefore, studies on the synthesis and antitumor activities of 6-isomers of 5, 8-*O*-dimethyl shikonin derivatives could be crucial for deriving a novel class for promising anti-cancer scaffolds.

Additionally, previous studies demonstrated that acylation of 1′-hydroxyl contained in the side chain of DMNQ derivatives potentiated the cytotoxic activity due to the enhancing effect of acylating on the electrophilicity of the quinonoid unit [6,10,11], thus raising renewed interest in the induction of the various organic acids to the hydroxyl group at the 1′ -position of 6-isomer of 5, 8-*O*-dimethyl shikonin as an available approach to producing prospective anti-tumor candidates. To testify the feasibility of this line of reasoning as well as to provide access to diverse analogues of the positional isomers, it would be useful to develop a synthetic method that allowed 6-isomers of 5,8-*O*-dimethyl acylshikonin derivatives, 1-(1,4-dihydro-5,8- dimethoxy-1,4- dioxonaphthalen -6-yl) -4-methylpent -3-enyl carboxylates, to be synthesized expediently. So in the study, we designed and prepared a series of 6-isomers of 5, 8-*O*-dimethyl acylshikonin derivatives starting from shikonin to give insight into their cytotoxic activities in vitro so as

* Corresponding author. Tel./fax: +86 21 34204775.
E-mail addresses: wzhou60@sjtu.edu.cn (W. Zhou), ssli@sjtu.edu.cn (S.-S. Li).
[1] Wen Zhou, Xu Zhang contributed equally to this work.

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doi:10.1016/j.ejmech.2011.05.006

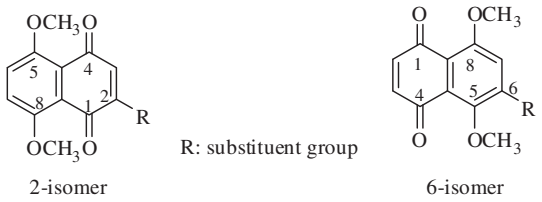


Fig. 1. Structures of 2-isomer and 6-isomer.

to acquire more potent and selective agents, and ultimately further evaluated for the antitumor activities on S-180 xenograft models with a pair of the positional isomers **5p**, **6c** as a representative example.

2. Chemistry

The published synthesis of 2-isomer of 5, 8-*O*-dimethyl shikonin (the same structure of 5, 8-*O*-dimethyl shikonin mentioned in reference 6.) was not suitable for the preparation of the key intermediate **4** [6]. Consequently, a novel synthetic pathway was needed to be established in order to prepare the derivatives of compound **4**. The preparation of 6-isomers of 5, 8-*O*-dimethyl acylshikonin derivatives (**5a-5v**) is outlined in Scheme 1.

Shikonin **1**, prepared in bulk according to the same procedure reported previously [6,12], was converted to 5,8-*O*-dimethoxymethyl shikonin **2** by treatment of newly distilled chloromethyl ether with anhydrous K_2CO_3 in DMF at 40 °C. Subsequently, synthesis of compound **3** was performed in accordance with the modified standard procedure of reductive methylation proposed by George [13], which was involved in treating compound **2** with sodium hydrosulfite in the mixture of THF and water as the ratio of 4: 1, and followed by adding sodium hydroxide and dimethyl sulfate and heating under the atmosphere of N_2. Noteworthy, the catalytic amount of tetrabutylammonium bromide was crucial for the yield of compound **3** [14].

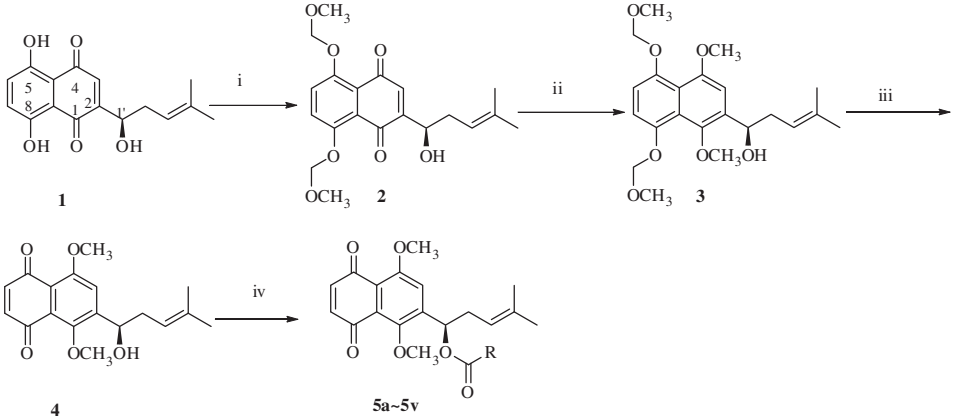
Next, the removal of methoxymethyl groups was conducted smoothly by means of 10% HCl [15] and further exposed to air to afford compound **4**, 6-isomer of 5,8-*O*-dimethyl shikonin, in 65.5% yield. The structure of the resulting product was explained distinctly that the chemical shifts of hydrogens on the naphthoquinone ring were used to determine the detailed difference with 2-isomers of 5,8-*O*-dimethyl shikonin. In fact, the significant difference was attributed to the presence of the substituent position of the side chain, which resulted in the differentiation of three hydrogens of naphthoquinone displayed in ^{1}H-NMR. Generally speaking, two hydrogens of benzene ring of 2-isomers of 5,8-*O*-dimethyl shikonin and its derivatives appeared at lower field than one hydrogen of their benzoquinone, while two hydrogens of benzoquinone of all 6-isomers indicated at the relatively higher field than one hydrogen of their benzene ring.

Finally, the target compounds **5a**–**5v** derived from the corresponding organic acids by treatment of dicyclohexylcarbodiimide (DCC) and dimethylaminopyridine (DMAP) with compound **4** at room temperature overnight [16].

3. Results and discussion

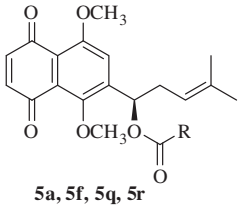
The in vitro cytotoxic activity of all the compounds prepared against the cancerous cells and normal cells was evaluated by the cell-based MTT Assay [6,17] using shikonin as a positive control. Antitumor potency of the tested derivatives was showed by IC_{50} values that were calculated by linear regression analysis of the concentration-response curves afforded for each compound. The data are described in Table 1 and Table 2. Afterwards, two typical positional isomers (**5p**, **6c**) were further evaluated with an S-180 xenograft model in male KM mice.

To identify whether the position of the side chain linked to DMNQ was related to the antitumor activity, we compared the cytotoxicities of several 2-isomers of 5,8-*O*-dimethyl acylshikonin derivatives, which possessed the remarkably effective antitumor activities among the tested compounds [6], with their corresponding 6-isomers against K562 (leukemia), MCF-7 (breast cancer), HCT-15(colon cancer), CNE (nasopharyngeal cancer), MDA-MB-231(breast cancer), L-929 (mouse fibroblast growth) and HSF(human skin fibroblasts). As indicated in Table 1, all the compound tested were effective against five tumorigentic cell lines with the IC_{50} values ranging from 0.2 μM to 58.0 μM. Compound **5a, 5f, 5p, 5r** exhibited 2–9-fold more active and selective toward MDA-MB-231 and MCF-7 cell lines than the corresponding isomers **6a**–**6d**, and the tendency was in good accordance with the relationship of 6-isomers and 2-isomers of DMNQ analogues against L1210 cell [7,8]. The greater activities of 6-isomers than 2-isomers were due to the introduction of the same side chain with an ester group into different positions of 6- and 2-isomers and the exposure of quinone moiety as a Michael acceptor to cellular nucleophiles [9,18]. Although compound **6a**–**6d** were shown to have slightly more effective on K562, CNE and HCT-15 cell lines than those of the corresponding 6-isomers except compound **6a** against K562, on the whole the inhibitory activities to these cell lines decreased significantly than the lead compound shikonin. Therefore, a great difference between IC_{50} values of the two positional isomers

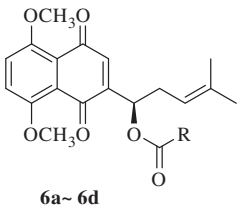


Scheme 1. Reagents and conditions: (i) CH_3OCH_2Cl/K_2CO_3/DMF, 1 h, 40 °C, N_2; (ii) $Na_2S_2O_4/(CH_3)_2SO_4$/NaOH/N(But)$_4$Br/THF/H_2O, 2h, reflux; (iii) *i*-PrOH/THF/10%HCl, r.t. overnight; (iv) DCC/DMAP/CH_2Cl_2/RCOOH, r.t, overnight.

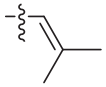
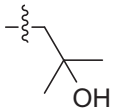
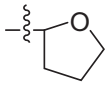
Table 1
Cytotoxicity comparision of 6- and 2- isomers of 5,8-*O*-dimethyl acylshikonin derivatives against K562, MCF-7, HCT-15, CNE, MDA-MB-231, L-929, HSF.



5a, 5f, 5q, 5r

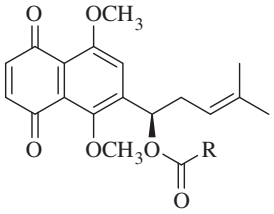


6a~ 6d

Compound	R	Tumorigenic cell lines $IC_{50}(\mu M)$					Normal cell lines $IC_{50}(\mu M)$	
		K562	MCF-7	HCT-15	CNE	MDA-MB-231	L-929	HSF
5a		15.3	4.9	19.3	58.0	3.4	>100	>100
6a		18.2	13.4	29.6	23.1	7.2	>100	87.5
5f		16.8	0.9	21.9	49.2	1.7	>100	>100
6b		11.7	3.3	19.5	27.6	6.8	>100	>100
5p		17.6	0.35	16.4	46.2	0.2	>100	>100
6c		15.3	2.2	13.1	32.1	1.7	>100	>100
5r		11.2	2.5	18.6	20.6	0.6	>100	>100
6d		11.5	5.7	12.7	16.5	2.1	>100	>100
Shikonin		0.6	1.9	7.3	0.7	6.6	3.2	1.3

The data of antitumor activities of compound 6a ~ d came from reference 6; Mean IC_{50} values were calculated from at least three independent experiments.

Table 2
Cytotoxicity of 6- isomers of 5,8-*O*-dimethyl acylshikonin derivatives against MDA-MB-231, MCF-7, HSF.



5a~5v

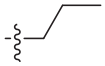
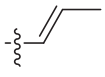
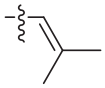
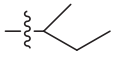
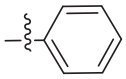
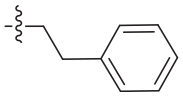
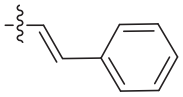
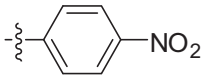
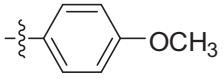
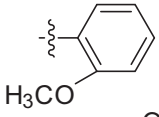
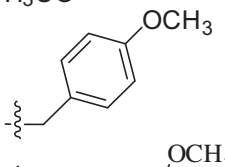
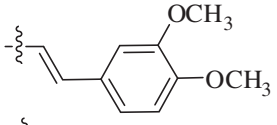
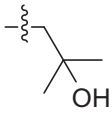
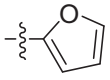
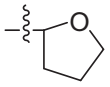
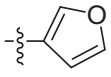
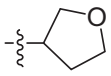
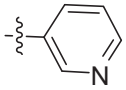
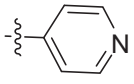
Compound	R	IC_{50} (μM)		
		MDA-MB-231	MCT-7	HSF
5a		3.4	4.9	>100
5b		2.5	6.2	78.6
5c		4.3	5.1	86.3
5d		2.8	3.7	69.3
5e		1.5	3.3	74.9
5f		1.7	0.9	>100
5g		5.9	4.5	93.6

Table 2 (*continued*)

Compound	R	IC_{50} (μM)		
		MDA-MB-231	MCT-7	HSF
5h		2.2	6.7	>100
5i		3.7	5.9	>100
5j		2.4	3.9	>100
5k		16.8	15.2	>100
5l		1.4	8.3	88.3
5m		4.4	21.3	>100
5n		2.3	5.5	>100
5o		5.0	7.7	>100
5p		0.2	0.35	>100
5q		1.3	2.7	>100
5r		0.6	2.5	>100
5s		0.4	1.3	>100
5t		0.2	1.1	69.4
5u		6.0	17.4	91.7
5v		1.2	6.3	>100
4		4.1	9.2	79.6
1	shikonin	6.6	1.9	1.3

Mean IC_{50} values were calculated from at least three independent experiments.

Table 3
Tumor growth inhibition in KM mice implanted with S-180 sarcoma.

Group	Dose (mg/kg)	Routes	Numbers		BWC (%)	TW(g) mean ± SD	TWI (%)
			D_0	D_{10}			
Control		i.p.	10	10	+39.1%	2.07 ± 0.42	
5p	3	i.p	10	10	+32.6%	1.24 ± 0.29**	40.0
5p	6	i.p	10	10	+25.3%	0.91 ± 0.07**	55.9
5p	12	i.p.	10	10	+20.5%	0.58 ± 0.10**	72.4
6c	12	i.p.	10	10	+22.9%	0.89 ± 0.08**	57.1
5-FU	25	i.p.	10	10	+34.7%	1.11 ± 0.05**	46.5

Data were presented as $\overline{X} \pm$ SD, and significance was assessed with Student's *t*-test. Differences were considered significant at $^{**}p < 0.01$, compared with control group; D_0 means the day before beginning to dose.; D_{10} means the 10th day; BWC means average body weight change; TW average means tumor weight; TWI means the average inhibition rate of tumor weight.

implied that diverse mechanisms need to be considered to explain the cytotoxicity against measured cancer cell lines, and that the importance of the position of the side chain connected to DMNQ had a profound influence on the antitumor activities. It was noticeable that the tested 6-isomers of 5,8-*O*-dimethyl acylshikonin derivatives (**5a, 5f, 5p, 5r**) also displayed no cytotoxicity to the non-tumerigentic cell lines (IC_{50} > 100 μM) and retained the same selectivity to breast cancer cells as most of 2-isomers reported previously [6]. Taken together, besides the significant increase of the selectivity, the 6-isomers of 5,8-*O*-dimethyl acylshikonin derivatives were comparative to, or more active against MDA-MB-231 and MCF-7 cell lines than the reference compound shikonin, and thus it was suggested that 6-isomer of 5,8-*O*-dimethyl shikonin **4** was a useful lead compound for the synthesis of naphthoquinone derivatives against certain specific cancer cell types.

As it was proved that the introduction of various substituents to 1′-position in the side chain of shikonin was closely related with an increase in cytotoxicity [6], it was appealing that more 6-isomers of 5,8-*O*-dimethyl acylshikonin derivatives were synthesized to undertake more indepth investigations into the cytotoxicities against selected MDA-MB-231, MCF-7, which turned out to be the most sensitive cell lines suppressed by **5a, 5f, 5p, 5r** and **6a**–**6d**. It had been observed from Table 2 that all the compounds but compound **5k, 5m, 5u** had higher cytotoxicities than compound **4** with IC_{50} values of less than 8.3 μM in breast cancer lines, implying that the positive effect of the attachment of various organic acids to the side chain of shikonin. The introduction of saturated alkylgroup to 1′-hydroxyl group in the side chain could benefit the increase of antitumor potency to some extent (**5a-5d**), and the unsaturated alkylgroups could further promote the enhancement of the cytotoxic activity (**5e, 5f**). As demonstrated in Table 2, compound **5f** was approximately 10-fold more active against MCF-7 with the IC_{50} values of 0.9 μM than compound **4** (IC_{50} = 9.2 μM). In addition, whether the ester containing heteroatom had a significant influence on the cytotoxicity to the target compounds against cancer lines. For instance, compound **5p** bearing hydroxyl group was found to be 5-fold and 33-fold more potent than the lead compound shikonin against corresponding MCF-7 (IC_{50} = 0.35 μM vs. IC_{50} = 1.9 μM) and MDA-DB-231 (IC_{50} = 0.2 μM vs. IC_{50} = 6.6 μM), and be more markedly potent than other tested compounds as well. It remained to be further studied whether there was a unique mechanism of action responsible for its high antitumor activities as the analogue of β-hydroxyisovaleryl-shikonin, an ATP-noncompetitive inhibitor of tyrosine kinases such as v-Src and EGFR [19]. There was similarity in compounds **5q**–**5t** bearing respective 2′-furyl, 2′-tetrahydrofuryl, 3′-furyl and 3′-tetrahydrofuryl to show stronger cytotoxic activity with the IC_{50} value of less than 1.3 μM against MDA-DB-231 than compound **4** (IC_{50} = 4.1 μM). Apparently, the relative positions and types of the heteroatom and whether it was saturated seemed to play an important part in the cytotoxic activity (**5q**–**5v**). In vitro evidence form Table 2 indicated that the switch of heteroatom' positions and the introduction of double bonds to the ring could lead to significant change of the cytotoxic activity.

Unlike our previous report [6,16], sharp drop in cytotoxic activity did not appear when replacing the alkylgroup with the aryl moiety (**5h**–**5o**) in cancer cell lines, suggesting that an increase in steric hindrance beneficial to the enhancement of the antitumor effects was associated probably with other mechanisms. Although the position of methoxy substituent on aryl moiety had not significant influence on the cytotoxicity, the para-position (**5l**) had an advantage over the ortho-position **(5m)** against the cancer lines. Replacement of the methoxy group with an electron-withdrawing nitro-group (**5k**) led to a dramatic decrease of antitumor potency against MDA-DB-231 compared with compound **5l**. Additionally, the phenethyl and cinnamenyl in place of the phenyl resulted in the corresponding compounds **5i**, **5j**, which had similar effects on the cytotoxic activity.

In order to compare the ability of 2-isomer of 5,8-*O*-dimethyl acylshikonin derivative **6c** to inhibit the tumor growth with that of its corresponding 6-isomer **5p** and their toxic effects in vivo test, KM mice bearing subcutaneous sarcoma S-180 were performed as an evaluation model [6], which shikonin derivatives had possessed good antitumor activities towards [20]. As demonstrated in Table 3, compound **5p, 6c** significantly inhibited the growth of sarcoma S-180 with 72.4%, 57.1% respectively when administered intraperitoneally (i.p.) at 12 mg/kg once a day for 9 days in comparison with the control group. However, 5-FU used as a positive control was showed only 46.5% suppression via i.p.at the dose of 25 mg/kg once day. Consistent with the previous report [6], 2-isomer of 5,8-*O*-dimethyl acylshikonin derivative **6c** at 12 mg/kg showed the similar inhibition rate of 57.1%, which was comparative to the antitumor activity of the corresponding isomer **5p** at 6 mg/kg. From Table 3 the dose-dependent relationship was observed in the tumor growth suppressed by compound **5p**. Treatment of the model mice with compound **5p** at 3 mg/kg, 6 mg/kg and 12 mg/kg resulted in the inhibitory effects of 40.0%, 55.9%, 72.4% respectively, which positively related with concentrations. It was noteworthy that no significant toxicity was found in two dimethylated shikonin derivatives-treated mice. Evidences from the antitumor effects in vivo indicated the two positional derivatives were more potent antitumor agents than the positive control, and 2-isomer of 5,8-*O*-dimethyl acylshikonin derivative **6c** less active than its 6-isomer **5p**, further reflecting the fact that the position of the side chain attached to DMNQ was vital for enhancing the antitumor potency of shikonin derivatives.

4. Conclusion

In summary, we have designed and synthesized twenty-two 6-isomers of 5, 8-*O*-dimethyl acylshikonin derivatives, and evaluated for their antitumor effects using two susceptible cancer cells

and the normal cell and KM mice implanted S-180 carcinoma subcutaneously. Most of the prepared compounds displayed the selective cytotoxic activities toward breast cancer cells MCF-7, MDA-MB-231 together with no cytotoxicity to the normal cell, and they were also found to exhibit more active than or comparative to the positive control shikonin. More importantly, the in vitro and in vivo evidences unfolded that they achieved greater activities than the corresponding 2-isomers of 5, 8-*O*-dimethyl acylshikonin derivatives and displayed more potent than 5-Fu, a typical drug clinically. This study may provide a wealth of knowledge that the position of the side chain of shikonin attached to DMNQ, together with the introduction of an appropriate oxygen-containing group to the 1′-hydroxyl in the side chain of shikonin, hold great promise for future development as antitumor agents with higher selectivity and lower toxicity.

5. Experimental protocols

5.1. Chemistry

Reagents and solvents were commercially available. Solvents were dried and purified using standard techniques. All reactions involving air or moisture sensitive or intermediates were carried out under the atmosphere of nitrogen. Melting points were measured on an SGW X-4 micro-melting point apparatus and are uncorrected. NMR spectra were determined on Varian Mercury-300 spectrometer (300 MHz for ^{1}H and 75 MHz for ^{13}C), chemical shifts of ^{1}H and ^{13}C spectra were recorded with tetramethylsilane as internal standard. Mass spectra were recorded on a Shimadzu LCMS-2010EV mass spectrometer. Column chromatography was run on silica gel (200–300 mesh) from Qingdao Ocean Chemical Factory.

5.2. 2-(1-hydroxy-4-methylpent-3-enyl)-5,8-bis-(methoxymethoxy)-naphthalene-1,4-dione (**2**)

Shikonin **1** (2.88 g, 0.01 mol) and potassium carbonate(13.8 g, 0.1 mol) in dimethyl formamide (20 ml) were stirred for 30 min at 40 °C, and then newly distilled chloromethyl ether was added in batches. The reaction proceeded for 1 h under the monitoring of TLC. Ethyl acetate (30 ml) and distilled water (20 ml) were poured into the reaction mixture. The organic layer was washed by water and brine respectively, dried over anhydrous $MgSO_4$ for 1 h and concentrated in *vacuo*. The residual oil was purified by flash chromatography on silica gel with ethyl acetate and petroleum ether (V:V = 1:2) to afford 2-(1-hydroxy-4-methylpent-3-enyl)-5,8-bis-(methoxymethoxy)- naphthalene-1,4-dione **2** (2.24 g, 77.1%) as yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.47 (s, 2H, benzene ring H), 6.80 (s, 1H, quinone ring H), 5.30 (m, 5H), 4.76 (t, 1H, J = 6.3 Hz), 3.56 (s, 6H), 2.55 (m, 2H), 1.72 (s, 3H), 1.62 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.1, 184.8, 152.6, 152.1, 136.7, 134.4, 125.6, 125.0, 120.4, 119.1, 102.6, 96.3, 96.2, 68.9, 57.9, 56.9, 56.8, 35.7, 26.1, 18.3. ESI-MS: 399.39 $(M + Na)^+$.

5.3. 1-[1,4-dimethoxy-5,8-bis-(methoxymethoxy)-naphthalen-2-yl]-4-methylpent-3-en-1-ol (**3**)

To a solution of 2-(1-hydroxy-4-methylpent-3-enyl)-5,8-bis-(methoxymethoxy)-naphthalene- 1,4-dione **2** (3.76 g, 0.01 mol) in THF (40 ml) and water (10 ml) was added sodium hydrosulfite (17.4 g, 0.1 mol) at room temperature under the atmosphere of N_2. When the color of the reaction solution turned into pale-yellow, sodium hydroxide (4.0 g, 0.1 mol) and tetrabutyl ammonium bromide (200 mg) was added successively, and then dimethyl sulfate (13.41 ml, 0.08 mol) was syringed at 40 °C, After being stirred for 2 h under refluxing, the reaction mixture was poured into iced water (100 ml), and then extracted with dichloromethane in twice (200 ml). The combined organic phase was dried over anhydrous $MgSO_4$, afterwards filtrated, and concentrated in *vacuo*. The residues were chromatographed on a silica gel column with ethyl acetate/petroleum ether (v/v: 1/4) to give compound **3** (2.86 g, 70.4%) as pale-orange oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.47 (m, 3H, benzene ring H), 5.28 (m, 6H), 3.94 (s, 6H), 3.57 (s, 6H), 2.61 (m, 2H), 1.74 (s, 3H), 1.68 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 153.3, 149.5, 148.9, 146.4, 135.6, 134.0, 120.4, 116.2, 115.2, 105.7, 98.2, 97.7, 68.7, 63.0, 57.1, 56.7, 56.6, 37.6, 26.1, 18.2. ESI-MS: 429.46 $(M + Na)^+$.

5.4. 6-(1-hydroxy-4-methylpent-3-enyl)-5,8-dimethoxynaphthalene-1,4-dione (**4**)

10% hydrochloric acid (10 ml) was added to a solution of 1-[1, 4-dimethoxy-5,8-bis -(methoxymethoxy)-naphthalen-2-yl]-4-methylpent-3-en-1-ol **3** (2.03 g, 0.005 mol) in the mixture of THF(20 ml) and isopropanol (5 ml), and the reaction was stirred for 3 h under the monitor of TLC. Then the suitable amount of saturated $NaHCO_3$ and ethyl acetate (80 ml) were added respectively. The organic layer was washed with water (20 ml) and brine (40 ml), then dried by anhydrous Na_2SO_4, and filtrated, and then evaporated under the reduced pressure. The crude product was purified by silica gel column with ethyl acetate/petroleum ether (v/v: 1/2) to obtain the compound **4** (1.03 g, 65.5%) as orange oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.55 (s, 1H, benzene ring H), 6.79 (s, 2H, quinone ring H), 5.24 (m, 1H), 5.10 (t, 1H, J = 5.7 Hz), 3.97 (s, 3H), 3.89 (s, 3H), 2.35 (m, 2H), 1.76 (s, 3H), 1.65 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 185.1, 184.5, 156.5, 150.9, 147.9, 139.2, 137.9, 136.9, 125.1, 68.8, 62.4, 56.9, 37.2, 26.1, 18.2. ESI-MS: 339.13 $(M + Na)^+$.

5.5. General procedure for preparation of 1-(1,4-dihydro-5, 8-dimethoxy-1,4-dioxonaphthalene -6-yl) -4-methlypent-3-enyl caroxylates (**5a**–**5v**)

To 6-(1-hydroxy-4-methylpent-3-enyl)-5,8-dimethoxynaphthalene-1,4-dione **4** (0.1 mol) and carboxylic acid (0.15 mol) in anhydrous CH_2Cl_2 were added DCC (0.2 mol) and DMAP (0.05 mol). After stirring overnight at room temperature under nitrogen atmosphere, petroleum ether was added to the reaction mixture to facilitate precipitates, and then the solution was filtered, and concentrated in *vacuo*. The residual oil was purified by flash chromatography to give **5a**–**5v** as yellow oil.

5.5.1. 1-(1,4-dihydro-5,8-dimethoxy -1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl acetate (**5a**)

Yield 73.7%, yellow oil. ^{1}HNMR (300 MHz, $CDCl_3$): δ 7.26 (s, 1H, benzene ring H), 6.79 (s, 2H, quinone ring H), 6.16 (t, 1H, J = 7.8 Hz), 5.11 (t, 1H, J = 7.5 Hz), 3.97 (s, 3H), 3.82 (s, 3H), 2.63 (m, 2H), 2.10 (s, 3H), 1.68 (s, 3H), 1.52 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 184.6, 184.1, 169.8, 155.9, 150.5, 144.3, 138.8, 137.7, 135.6, 125.1, 120.0, 118.0, 116.7, 70.4, 61.9, 56.6, 33.9, 25.5, 21.0, 17.7. ESI-MS: 381.14 $(M + Na)^+$.

5.5.2. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl propionate (**5b**)

Yield 54.3%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.25 (s, 1H, benzene ring H), 6.77 (s, 2H, quinone ring H), 6.11 (t, 1H, J = 7.2 Hz), 5.14 (t, 1H, J = 7.2 Hz), 3.98 (s, 3H), 3.91(s, 3H), 2.55 (m, 4H), 1.68 (s, 3H), 1.53 (s, 3H), 1.15 (t, 3H, J = 6.9 Hz). ESI-MS: 395.15 $(M + Na)^+$.

5.5.3. 1-(1,4-dihydro-5,8-dimethoxy -1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl butyrate (**5c**)

Yield 50.1%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.24 (s, 1H, benzene ring H), 6.77 (s, 2H, quinone ring H), 6.16 (t, 1H,

J = 7.8 Hz), 5.12 (t, 1H, J = 5.4 Hz), 3.95 (s, 3H), 3.90 (s, 3H), 2.47 (m, 4H), 1.71 (m, 5H), 1.62 (s, 3H), 0.98 (t, 3H, J = 7.5 Hz). ESI-MS: 409.15 (M + Na) $^+$.

*5.5.4. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl isobutyrate (**5d**)*

Yield 57.8%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.24 (s, 1H, benzene ring H), 6.77 (s, 2H, quinone ring H), 6.10 (t, 1H, J = 5.1 Hz), 5.13 (m, 1H), 3.94 (s, 3H), 3.92 (s, 3H), 2.66 (m, 3H), 1.49 (s, 3H), 1.47 (s, 3H), 1.21 (d, 3H, J = 2.1 Hz), 1.16 (d, 3H, J = 2.1 Hz). ESI-MS: 409.15 (M + Na) $^+$.

*5.5.5. (E)-1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl but-2-enoate (**5e**)*

Yield 47.7%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.24 (s, 1H, benzene ring H), 7.04 (m, 1H), 6.78 (s, 2H, quinone ring H), 6.17 (m, 1H), 5.94 (m, 1H), 5.12 (t, 1H, J = 7.8 Hz), 3.96 (s, 3H), 3.93 (s, 3H), 2.56 (m, 2H), 1.93 (m, 3H), 1.67 (s, 3H), 1.53 (s, 3H). ESI-MS: 407.17 (M + Na) $^+$.

*5.5.6. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl 3-methylbut-2-enoate (**5f**)*

Yield 56.9%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.24 (s, 1H, benzene ring H), 6.78 (s, 2H, quinone ring H), 5.90 (m, 1H), 5.43 (s, 1H), 5.13(m, 1H), 3.94 (s, 3H), 3.92 (s, 3H), 2.53 (m, 2H), 2.15 (s, 3H), 1.93 (s, 3H), 1.65 (s, 3H), 1.54 (s, 3H). ESI-MS: 421.16 (M + Na) $^+$.

*5.5.7. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl 2-methylbutanoate (**5g**)*

Yield 42.7%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.24 (d, 1H, J = 1.5 Hz, benzene ring H), 6.77 (s, 2H, quinone ring H), 6.10 (m, 1H), 5.13 (t, 1H, J = 5.4 Hz), 3.93 (s, 3H), 3.92 (s, 3H), 2.64 (m, 3H), 1.63 (s, 3H), 1.52 (s, 3H), 1.19 (m, 2H), 1.16 (m, 6H). ESI-MS: 423.18 (M + Na) $^+$.

*5.5.8. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl benzoate (**5h**)*

Yield 27.2%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.08 (m, 2H, benzene ring H), 7.61 (m, 3H, benzene ring H), 7.32 (s, 1H, benzene ring H), 6.74 (s, 2H, quinone ring H), 6.17 (m, 1H), 5.21 (t, 1H, J = 6.9 Hz), 4.01 (s, 3H), 3.97 (s, 3H), 2.72 (m, 2H), 1.66 (s, 3H), 1.59 (s, 3H). ESI-MS: 443.15 (M + Na) $^+$.

*5.5.9. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl 3-phenylpropanoate (**5i**)*

Yield 31.9%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ7.29 (m, 6H, benzene ring H), 6.78 (s, 2H, quinone ring H), 6.17 (m, 1H), 5.10 (t, 1H, J = 7.5 Hz), 3.90 (s, 3H), 3.87 (s, 3H), 2.99 (t, 2H, J = 7.8 Hz), 2.75 (t, 2H, J = 7.8 Hz), 2.53 (m, 2H), 1.67 (s, 3H), 1.52 (s, 3H). ESI-MS: 471.18 (M + Na) $^+$.

*5.5.10. 1-(1,4 -dihydro-5,8-dimethoxy -1,4-dioxoaphthalent-6-yl)-4-methypent-3-enyl cinnamate (**5j**)*

Yield 35.6%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.75 (d, 1H, J = 16.2 Hz), 7.58 (m, 6H, benzene ring H), 6.79 (s, 2H, quinone ring H), 6.55 (d, 1H, J = 16.2 Hz), 6.25 (dd, 1H, J = 7.5, 4.8 Hz), 5.19 (t, 1H, J = 6.0 Hz), 3.96 (s, 3H), 3.94 (s, 3H), 2.57 (m, 2H), 1.69 (s, 3H), 1.56 (s, 3H). ESI-MS: 469.16 (M + Na) $^+$.

*5.5.11. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl 4-nitrobenzoate (**5k**)*

Yield 68.3%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.35 (m, 4H, benzene ring H), 7.29 (s, 1H, benzene ring H), 6.80 (s, 2H, quinone ring H), 6.40 (m, 1H), 5.20 (t, 1H, J = 7.2 Hz), 3.98 (s, 3H), 3.90 (s, 3H), 2.70 (m, 2H), 1.69 (s, 3H), 1.59 (s, 3H). ESI-MS: 488.13 (M + Na) $^+$.

*5.5.12. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl 4-methoxybenzoate (**5l**)*

Yield 36.8%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.10 (d, 1H, J = 9.0 Hz, benzene ring H), 8.09 (d, 1H, J = 9.0 Hz, benzene ring H), 7.31 (s, 1H, benzene ring H), 6.99 (d, 1H, J = 9.0 Hz, benzene ring H), 6.98 (d, 1H, J = 9.0 Hz, benzene ring H), 6.78 (s, 2H, quinone ring H), 6.31 (m, 1H), 5.23 (t, 1H, J = 6.9 Hz), 3.97 (s, 3H), 3.88 (s, 3H), 3.83 (s, 3H), 2.68 (m, 2H), 1.67 (s, 3H), 1.56 (s, 3H). ESI-MS: 473.16 (M + Na) $^+$.

*5.5.13. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl 2-methoxybenzoate (**5m**)*

Yield 35.9%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.86 (m, 1H, benzene ring H), 7.53 (m, 1H, benzene ring H), 7.41 (s, 1H, benzene ring H), 7.04 (m, 2H, benzene ring H), 6.77 (d, 2H, J = 1.2 Hz, quinone ring H), 6.32 (m, 1H), 5.24 (t, 1H, J = 6.9 Hz), 3.98 (s, 3H), 3.93 (s, 3H), 3.90 (s, 3H), 2.67 (m, 2H), 1.68 (s, 3H), 1.52 (s, 3H). ESI-MS: 473.16 (M + Na) $^+$.

*5.5.14. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl 2-(4-methoxyphenyl)acetate (**5n**)*

Yield 33.4%, yellow oil, ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.25 (d, 2H, J = 8.1 Hz, benzene ring H), 6.93 (s, 1H, benzene ring H), 6.87 (d, 2H, J = 8.1 Hz, benzene ring H), 6.53 (s, 2H, quinone ring H), 6.11 (m, 1H), 5.13 (t, 1H, J = 8.4 Hz), 3.91 (s, 3H), 3.88 (s, 3H), 3.73 (s, 3H), 3.63 (s, 2H), 2.55 (m, 2H), 1.67 (s, 3H), 1.52 (s, 3H). ESI-MS: 487.17 (M + Na) $^+$.

*5.5.15. (E)-1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl 3-(3,4-dimethoxyphenyl)acrylate (**5o**)*

Yield 33.7%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.67 (d, 1H, J = 15.9 Hz,), 7.29 (s, 1H, benzene ring H), 7.67 (d, 1H, J = 8.7 Hz, benzene ring H), 7.06 (s, 1H, benzene ring H), 7.67 (d, 1H, J = 8.7 Hz, benzene ring H), 6.37 (s, 2H, quinone ring H), 6.34 (d, 1H, J = 15.6 Hz), 6.22 (m, 1H), 5.20 (t, 1H, J = 6.0 Hz), 3.95 (s, 3H), 3.94 (s, 3H), 3.93 (s, 3H), 3.81 (s, 3H), 2.61 (m, 1H), 2.37 (m, 1H), 1.68 (s, 3H), 1.58 (s, 3H). ESI-MS: 529.18 (M + Na) $^+$.

*5.5.16. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl 2-hydroxy-2-methylpropanoate (**5p**)*

Yield 45.5%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.27 (s, 1H, benzene ring H), 6.67 (s, 2H, quinone ring H), 6.18 (m, 1H), 5.04 (t, 1H, J = 8.4 Hz), 3.95 (s, 3H), 3.94 (s, 3H), 2.58 (m, 4H), 1.68 (s, 3H), 1.55 (s, 3H), 1.29 (s, 3H), 1.26 (s, 3H). ESI-MS: 439.17 (M + Na) $^+$.

*5.5.17. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl furan-2-carboxylate (**5q**)*

Yield 49.2%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.69 (m, 1H, furan ring), 7.34 (s, 1H, benzene ring H), 7.25 (m, 1H, furan ring H), 6.79 (s, 2H, quinone ring H), 6.56 (m, 1H, furan ring H), 6.34 (t, 1H, J = 5.1 Hz), 5.10 (t, 1H, J = 2.7 Hz), 3.96 (s, 3H), 3.92 (s, 3H), 2.67 (m, 2H), 1.68 (s, 3H), 1.57 (s, 3H). ESI-MS: 433.13 (M + Na) $^+$.

*5.5.18. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl tetrahydrofuran-2-carboxylate (**5r**)*

Yield 42.8%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.28 (d, 1H, J = 3.3 Hz, benzene ring H), 6.79 (s, 2H, quinone ring H), 6.12 (m, 1H), 5.14 (t, 1H, J = 6.3 Hz), 4.55 (t, 1H, J = 4.8 Hz), 4.02 (m, 8H), 2.58 (m, 2H), 2.25 (m, 1H), 2.08 (m, 3H), 1.68 (s, 3H), 1.55 (s, 3H). ESI-MS: 437.16 (M + Na) $^+$.

*5.5.19. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl furan-3- carboxylate (**5s**)*

Yield 49.2%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.10 (d, 1H, J = 1.2 Hz, furan ring H), 7.49 (d, 1H, J = 1.2 Hz, furan ring H), 7.29 (s, 1H, benzene ring H), 6.82 (s, 2H, quinone ring H), 6.80 (s, 1H, furan

ring H), 6.52 (dd, 1H, $J = 4.8, 4.8$ Hz), 5.19 (t, 1H, $J = 7.5$ Hz), 3.97 (s, 3H), 3.94 (s, 3H), 2,63 (m, 2H), 1.69 (s, 3H) 1.58 (s, 3H). ESI-MS: 433.13 $(M + Na)^+$.

*5.5.20. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl tetrahydrofuran-3-carboxylate (**5t**)*

Yield 57.4%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.24 (d, 1H, $J = 3.3$ Hz, benzene ring H), 6.78 (s, 2H, quinone ring H), 6.16 (m, 1H), 5.11 (t, 1H, $J = 6.3$ Hz), 4.02 (m, 10H), 3.19 (m, 1H), 2.53 (m, 2H), 2.24 (m, 2H), 1.68 (s, 3H), 1.54 (s, 3H). ESI-MS: 437.16 $(M + Na)^+$.

*5.5.21. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl nicotinate (**5u**)*

Yield 47.6%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 9.28 (s, 1H,pyridine ring H), 8.81 (d, 1H, $J = 3.6$ Hz, pyridine ring H), 8.33 (m, 1H,pyridine ring H), 7.46 (d, 1H, $J = 3.6$ Hz, pyridine ring H), 7.31 (s, 1H, benzene ring H), 6.79 (s, 2H, quinone ring H), 6.41 (m, 1H), 5.20 (t, 1H, $J = 7.2$ Hz), 3.97 (s, 3H), 3.94 (s, 3H), 2.76 (m, 2H), 1.66 (s, 3H) 1.59 (s, 3H) ESI-MS: 444.15$(M + Na)^+$.

*5.5.22. 1-(1,4-dihydro-5,8-dimethoxy-1,4-dioxonaphthalen-6-yl)-4-methylpent-3-enyl isonicotinate (**5v**)*

Yield 59.7%, yellow oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.84 (d, 2H, $J = 4.5$ Hz,pyridine ring H), 7.88 (d, 2H, $J = 4.5$ Hz,pyridine ring H), 7.28 (s, 1H, benzene ring H), 6.80 (s, 2H, quinone ring H), 6.39 (m, 1H), 5.19 (m, 1H), 3.97 (s, 3H), 3.90 (s, 3H), 2.70 (m, 2H), 1.69 (s, 3H), 1.58 (s, 3H). ESI-MS: 444.15 $(M + Na)^+$.

5.6. Antitumor activity in vitro

5.6.1. Cell culture

HCT-15, NCE, MDA-DB-231, MCF-7, K562, L-929 and HSF were grown in RPMI-1640 or Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 mM nonessential amino acids, 0.1 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

5.6.2. Assessment of antitumor activity by MTT assay

The compounds tested were dissolved in suitable amount of dimethyl-sulfoxide (DMSO) before the experiment to obtain the solution having the known concentration, from which a definite amount was taken and diluted to the various concentrations with nutrient solution. The concentration of HCT-15, NCE, MDA-DB-231, MCF-7, K562, L-929 and HSF cells was adjusted to 5.0×10^4 cells/mL respectively. Cells were seeded on 96-well plates. After cultured for 24 h in 37 °C humidified incubator (5% CO_2), cells were incubated in complete mediums with the absence (negative control) and presence of various concentrations of compounds tested for 24 h, respectively. Each group was arranged four parallel wells. The supernatant was removed, and then 20 μL of MTT solution (5 mg/mL) was added to each well. After re-incubated for another 4 h, 100 μL of DMSO was added to each well for dissolving the formazan crystals. The percentage of cell viability was determined by measuring the absorbance (Abs) at $\lambda = 570$ nm using a Multiskan MK3 microplate reader (Thermo, USA). Survival percentage was calculated using the following equation: inhibitory rate $= (Abs_{570\text{control cells}} - Abs_{570\text{ treated cells}})/Abs_{570\text{control cells}} \times 100\%$. IC_{50} values were obtained from linear regression analysis of the concentration-response curves plotted for each compounds tested.

5.7. Antitumor activity in vivo

Sarcoma S-180 cells (2×10^6) in 0.2 ml of physiological saline were subcutaneously injected between the femur of the male KM mice (Seven-week-old specific pathogen free (SPF) male KM mice with the weight of 18–22 g each were obtained from Shanghai Laboratory Animal Center, Chinese Academy of Sciences).Tumor-bearing mice were randomly subdivided into 5 groups of 10. After 24 h, mice were treated with compound **5p** via an intraperitoneal (i.p.) injection at a dose of 3, 6, 12 mg/kg once a day for 9 days (9 times) respectively while compound **6c** was administrated with 12 mg/kg. Similarly, 5-fluorouracil (5-FU, 25 mg/kg, i.p.) was administered as a positive control. It was noticeable that all compound tested were dissolved in 0.5% DMSO and 1% Tween 80, and diluted to the given concentration by physiological saline. The body weight of mice was observed. Subsequently mice were killed, and the tumors were excised and weighed in day 10. Control mice were injected with 0.5% DMSO and 1% Tween 80 in physiological saline (vehicle). Tumor growth inhibition was calculated as T/C (Treatment/control) by the following formula: $T/C = [(\sum C_{10}/10 - \sum T_{10}/10)/(\sum C_{10}/10)] \times 100\%$, where C_{10} is the tumor weight at day 10 in control group and T_{10} is the tumor weight at day 10 in treated group.

Acknowledgments

We thank National Natural Science Foundation of China (No.30973604, No.91013012) and Shanghai Natural Science Fund (No.11ZR1416300) for financial supports.

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