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Synthesis and evaluation of unsymmetrical polyamine derivatives as antitumor agents

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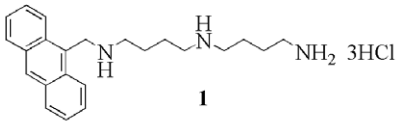
ABSTRACT

A series of unsymmetrically substituted polyamine derivatives were prepared and their cytotoxicities in mouse leukemia L1210, melanoma B16, and HeLa cells were investigated. The in vitro cytotoxicity revealed that these conjugates could recognize the polyamine transporter, and the *N*-ethyl modified homospermidine moiety may be another efficient carrier as homospermidine even though the introduction of terminal alkyl groups led to reduced cytotoxicity in comparison with the un-substituted counterpart **1**. The ornithine decarboxylase and topoisomerase II inhibition experiments indicated that ODC and TOPO II were potential, but not unique targets of these conjugates. Furthermore, the in vivo antitumor activities illustrated that the representative conjugate **2f** and the homospermidine analogue **1** evidently inhibited the tumor growth and significantly increased the survival time of mice-bearing sarcoma 180 cells.

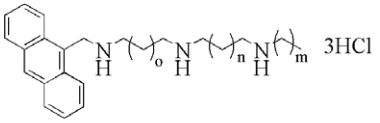
1. Introduction

During the past decades, the pathways for polyamine metabolism have been gradually elucidated and this has provided new avenues for research focusing on polyamine derivatives.[1–5] It is certainly well established that disturbance of cellular polyamine uptake will be a potent cancer chemotherapeutic strategy in drug development. In fact, highly proliferating cells (e.g., tumor cells) have up-regulated polyamine transport systems (PTS) for the uptake of exogenous polyamines.[6,7] Tumor cells with up-regulated PTS can accumulate polyamines more effectively than normal cells,[6–9] and the structural requirements of the PTS are not stringent although the precise mechanism has not been completely defined.[3,9] Thus, polyamine–drug conjugates, which structurally mimic the natural polyamines, will be readily ferried into cells by the polyamine transporter. These findings provide an attractive strategy using the polyamine backbone as a potential carrier for drug delivery. Previously, the non-native triamine, homospermidine, was reported to facilitate the uptake of relatively large N^1-aryl groups via the PTS.[10–13] In addition, Delcros et al. described that heterocyclic derivatives of various sizes once conjugated to a natural polyamine (putrescine, spermidine, and spermine) were able to be ferried via PTS, but had limitations in terms of the size of N^1-aryl substituent.[14] However, the bis-arylsubstituted diamine derivatives reported by Burns were shown to behave in a mode distinct from the polyamine analogues mentioned above.[15]

Previous work revealed that the N^1-anthracenylhomospermidine conjugate **1** (Fig. 1) exhibited a higher PTS recognition and selectivity in Chinese hamster ovary (CHO) cells.[10–12] Recent mechanistic studies indicated that **1** induced apoptosis in several cell



1



2a: m=1, n=2, o=1; **2e**: m=2, n=1, o=2
2b: m=1, n=1, o=2; **2f**: m=1, n=2, o=2
2c: m=2, n=1, o=1; **2g**: m=2, n=2, o=2
2d: m=2, n=2, o=1

Figure 1. Chemical structures of compounds **1** and **2a**–**g**.

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lines such as B16 cells and IL-3-dependent FL5.12A pro-B cells,[16,17] while its biological properties at whole animal level have been lacking. These findings prompted us to design novel polyamine skeletons as effective vectors.

In the course of conjugate generation, the anthracene, one DNA intercalator, was attached to the triamine architectures with various carbon chain lengths, and an alkyl group was introduced on the other terminal-N atom, with the hope that simple structural modification would be identified for additional metabolism and might exhibit better antitumor activity both in vitro and in vivo.[18] The anthracene was also chosen as the terminal substituent because of its accepted toxic response on cellular entry, which facilitated comparison of the potency of synthetic polyamine architectures.[10–12]

In this report, we describe the synthesis of a series of unsymmetrically substituted polyamine conjugates **2** (Fig. 1), the evaluation of their capability for PTS utilization, and their in vitro antitumor activity against several cancer cell lines. Furthermore, our investigations extended to other potential targets that may explain the drug's cytotoxicity such as ornithine decarboxylase (ODC) and Topoisomerase inhibition. In order to comprehend the in vivo anticancer activity of potent compounds, tumor growth inhibition and prolongation of survival time were investigated on tumor-bearing mice.

2. Results and discussion

2.1. Chemistry

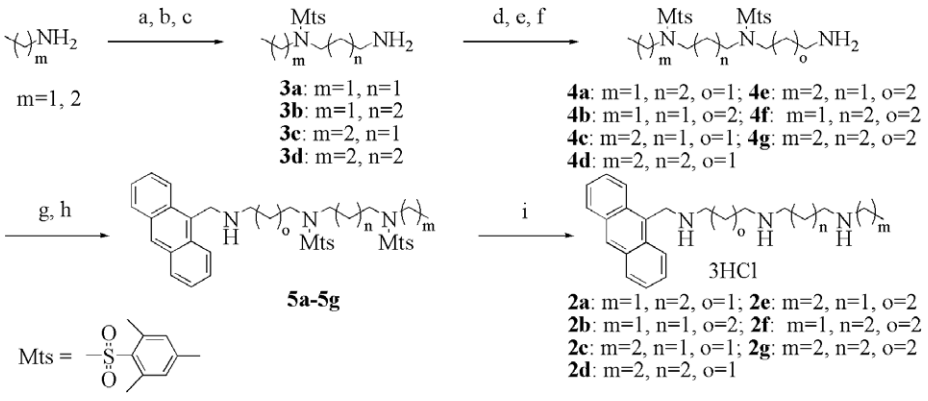
The synthesis of the unsymmetrically substituted polyamine derivatives involved the preparation of polyamine motifs and conjugation of these intermediates with 9-anthraldehyde. As shown in Scheme 1, the ordinary alkyl amines reacted with mesitylenesulfonyl chloride in a biphasic solution of CH_2Cl_2/NaOH gave the protected compounds, and then anion formation with NaH in DMF and coupling with *N*-bromoalkylphthalimide in DMF generated the phthalimide intermediate. Unmasking of the primary amino functionality with hydrazine hydrate in refluxing ethanol afforded the primary amines, **3a**–**d**. Using the same procedures described above provided the polyamine motif **4a**–**g**.

The targeted polyamine derivatives were prepared using the straightforward one-pot route shown in Scheme 1. The reductive amination of **4a–g** to **5a–g** was achieved in two steps by condensation of the primary amine with one equivalent of aldehyde and reduced by treatment with excess $NaBH_4$ in CH_2Cl_2/MeOH. Since purification of **5** was important, the intermediate was separated by flash column chromatography (chloroform/methanol 20:1), and immediately deprotected using 48% HBr in acetic acid and phenol in CH_2Cl_2 to give the hydrochloride salt **2a**–**g** (according to the previously reported procedures).[19] The structures of target compounds were confirmed by ^{1}H NMR, MS and elemental analysis, which were consistent with the proposed structures. The synthetic route turned out to be convenient and inexpensive in comparison with the amino alcohol strategy reported in the literature.[10] Also the control compound **1** was prepared to compare the cytotoxicity.

2.2. Cytotoxic activity

The in vitro cytotoxicities of the synthesized compounds were evaluated against mouse leukemia L1210, melanoma B16, and HeLa cells by MTT assays. Since many alkylpolyamine analogues induce spermidine/sperm-N^1-acetyltransferase (SSAT) and act as a substrate for SSAT and polyamine oxidase (PAO), toxic oxidation products produced in the metabolic pathway (e.g., aldehydes, H_2O_2) would add to the cytotoxic effect of the analogue.[20,21] Thus, experiments were run in the presence of aminoguanidine to avoid the disadvantages. In general, the unsymmetrically substituted polyamine derivatives **2a**–**g** exhibited significant to moderate cytotoxicity with IC_{50} values varying from 1.71–16.41 μM, but none of the seven compounds showed higher potency than the reference compound **1** (Table 1). However, inspection of the results revealed that compound **2f** displayed more potent inhibitory activity in HeLa and L1210 cells than the other conjugates tested. Interestingly, the homospermidine moiety containing an *N*-ethyl group is an important fragment existing in bis-ethyl polyamine derivatives.[2,3,20]

To investigate whether these conjugates could enter cells via PTS, the growth inhibitory effect was determined in B16 cells co-incubated with excess-free spermidine (SPD) or α-difluoromethylornithine (DFMO). Cells grown for 48 h with 500 μM SPD or 5 mM DFMO were exposed to various concentrations of the conjugates. Also, aminoguanidine (1 mM) was added to prevent oxidation of drugs by the enzyme present in the culture medium. As shown in Table 1, IC_{50} values showed moderate increases (r_1, ratio of IC_{50} values) for assays run in the presence of SPD. Interestingly, compounds **2b**, **2f**, and **2g** had higher levels of SPD protection than the control compound **1**. SPD, as reported, provides competition with polyamine conjugates for PTS utilization, and inhibits the uptake of structural analogues via the PTS in tumor cells.[22,23] The results illustrate that SPD protected the B16 cells from the conjugates tested and thus it can be rationalized that these conjugates com-



Scheme 1. Preparation of unsymmetrically substituted polyamine conjugates. Reagents and conditions: (a) MtsCl (1.1 equiv), NaOH, CH_2Cl_2/H_2O; (b) *N*-bromoalkylphthalimide, NaH, DMF; (c) $H_2NNH_2{\cdot}H_2O$, C_2H_5OH, reflux, 12 h; (d) MtsCl (1.1 equiv), NaOH, CH_2Cl_2/H_2O; (e) *N*-bromoalkylphthalimide, NaH, DMF; (f) $H_2NNH_2{\cdot}H_2O$, C_2H_5OH, reflux, 6 h; (g) 9-anthraldehyde, CH_2Cl_2/CH_3OH; (h) $NaBH_4$, CH_2Cl_2/CH_3OH; (i) 48% HBr/HOAc, phenol, NaOH, CH_2Cl_2, rt; HCl, C_2H_5OH, 0 °C.

Table 1
Cytotoxicity assay results for polyamine derivatives in HeLa, L1210, and B16 cells

Compound	HeLa IC_{50}, μM	L1210 IC_{50}, μM	B16 IC_{50}, μM	B16+SPD IC_{50}, μM	r_1^a IC_{50} ratio	B16+DFMO IC_{50}, μM	r_2^a IC_{50} ratio
1	1.19 ± 0.07	1.04 ± 0.06	1.40 ± 0.04	2.46 ± 0.03	1.76	0.97 ± 0.02	1.44
2a	10.36 ± 0.20	7.89 ± 0.13	7.02 ± 0.15	10.55 ± 0.15	1.50	2.73 ± 0.03	2.57
2b	13.95 ± 0.36	16.41 ± 0.36	7.74 ± 0.17	16.49 ± 0.14	2.13	2.70 ± 0.01	2.87
2c	11.71 ± 0.02	6.60 ± 0.09	5.43 ± 0.14	8.85 ± 0.25	1.63	1.02 ± 0.01	5.32
2d	10.15 ± 0.11	7.99 ± 0.12	5.93 ± 0.06	8.85 ± 0.19	1.49	1.99 ± 0.03	2.98
2e	11.09 ± 0.07	7.38 ± 0.17	4.05 ± 0.05	6.17 ± 0.06	1.52	1.31 ± 0.01	3.10
2f	2.26 ± 0.04	1.71 ± 0.09	5.08 ± 0.10	18.68 ± 0.55	3.68	2.69 ± 0.07	1.89
2g	7.16 ± 0.16	7.79 ± 0.17	9.66 ± 0.50	25.73 ± 0.35	2.66	4.50 ± 0.04	2.15

IC_{50} values from three-independent experiments were given as means ± SD.
r_1^a, r_2^a represent the ratio of IC_{50} values, (B16+SPD/B16) and (B16/B16+DFMO), respectively.

petitively penetrate into cells via the PTS. DFMO, which depletes intracellular polyamine level by inhibition of ornithine decarboxylase (ODC), could increase uptake of exogenous polyamines. Therefore, cells treated with DFMO should be more susceptible to polyamine analogues and should provide lower IC_{50} values. As a result, exposure of B16 cells to the conjugates in the presence of 5 mM DFMO enhanced their growth inhibitory activity (e.g., r_2 in Table 1). In DFMO-treated cells, IC_{50} values for **2a–g** were significantly lower when compared to their respective counterparts.[10] Interestingly, the synergistic effects of DFMO on these terminal alkyl-substituted derivatives were even stronger than that on the control **1**.[10–12] However, their cytotoxicity was attenuated by the introduction of terminal alkyl groups. This might be explained by the different intracellular targets effected by these drug types. With **2f** in hand, we evaluated how this simple structural modification of **1** influenced its biological behavior. In this regard, we evaluated their ODC and topoisomerase II inhibition activity. In short, N-ethylation had some interesting effects.

2.3. Ornithine decarboxylase inhibitory activity

For example, it is known that bis-ethyl polyamine analogues can inhibit the activity of ODC.[24] To assess the effect of selected conjugates on ODC activity, ODC 4293, (transformed human cells in which the expression of the ODC gene is greatly induced by estrogen), provided a negative control, while cells treated with DFMO (which inhibits ODC activity) provided the positive control.[25] The conjugates were tested at 5 μM and reduced ODC activity to different extents ranging from 33% to 56% compared with the negative control (Fig. 2). Compound **2g** exerted a higher inhibitory activity (about 67% inhibited compared to no treatment), while **1** reduced 31% of ODC activity, and DFMO, a direct ODC inhibitor, at 5 μM inhibited 90%. The results demonstrate the intricate influence of structural alterations on the pharmacological outcome.

2.4. Topoisomerase inhibitory activity

Topoisomerases are essential enzymes which regulate the topology and supercoiling of DNA in the course of transcription and replication. Topoisomerase I and II (TOPO I, TOPO II) can, respectively, catalyze single- and double-strand breakage during changes in the topological state of duplex DNA.[22,26,27] Consequently, these enzymes are important molecular targets of antineoplastic drugs.

To test whether our drugs influenced TOPO II activity, a simple electrophoretic assay[28] was used for assessing TOPO II mediated DNA relaxation activity. During electrophoresis, catenated kDNA has limited migratory ability and remains near the top well due to its high molecular weight, while its decatenated forms migrate further down the gel. As shown in Figure 3, when compounds **1** and **2f** were carried out at concentrations of 5, 10, and 20 μM, respectively, they all displayed strong TOPO II inhibitory activity. However, the terminal ethyl substituted anthracene–polyamine conjugate **2f** exhibited greater inhibitory activity than **1**. This difference is clearly observed at the concentration of 5 μM, wherein compound **1** (lane 8) markedly prevented kDNA from being decatenated by TOPO II, while compound **2f** completely inhibited the decatenation reaction with most of the kDNA remaining in the top well (lane 4). The TOPO I mediated DNA relaxation activity was also evaluated with the same compounds, but they did not show inhibitory activity at any of the applied concentrations (data

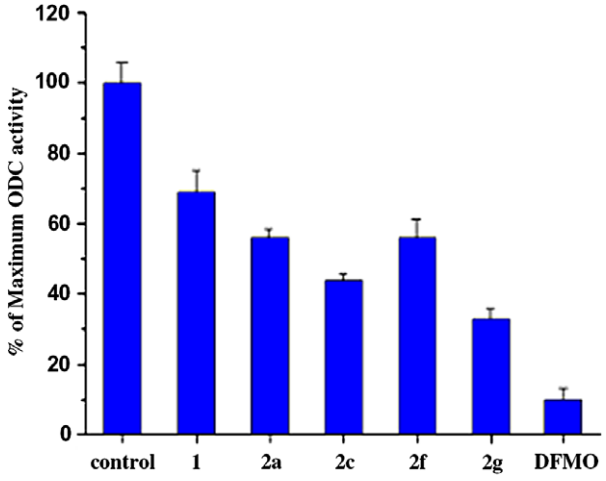


Figure 2. The effects of selected conjugates on ODC activity in ODC 4293 cells. Data from three separate experiments were given as means ± SD.

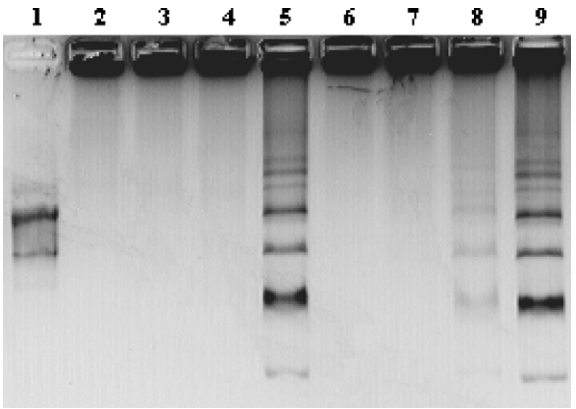


Figure 3. Effects of **1** and **2f** on the decatenation of kDNA by TOPO II, lane 1, TOPO II; lane 5, 9, kDNA with 1.5 U of TOPO II (decatenated form); lanes 2–4, kDNA with 1.5 U of TOPO II in the presence of **2f** at 20, 10, and 5 μM, respectively; lanes 6–8, kDNA with 1.5 U of TOPO II in the presence of **1** at 20, 10, and 5 μM, respectively.

not shown). The results are consistent with the TOPO II inhibitory capability of polyamine derivatives reported previously,[22] and indicate that the presence of the terminal alkyl group (e.g., ethyl) can enhance TOPO II inhibitory activity.

2.5. In vivo antitumor activity against subcutaneously transplanted sarcoma 180 cells

In vivo research has an advantage to discover drugs. Compound **1**, as reported previously,[10–12] had potent cytotoxicity against several tumor cell lines and exhibited a higher PTS recognition. However, its in vivo antitumor activity has not been reported till now. Therefore, compound **1** and **2f**, the representative conjugate above tested, were selected for a preliminary in vivo investigation. The acute maximum tolerated dose (MTD) was firstly determined following administration of increasing drug doses to healthy female Kunming mice. Therefore, **2f** and **1** were found to have a MTD of 20 mg/kg and 10 mg/kg, respectively.

To evaluate the effects of **2f** and **1** on tumor growth, Kunming mice inoculated with sarcoma 180 cells were treated with these analogues, and the tumor weights of mice were measured after 7 days of the administration. As shown in Table 2, preliminary results demonstrated promising tumor growth inhibition. For example, at the end of treatment, **2f** reduced tumor weight by 56.9% (0.69 ± 0.08 g), 44.4% (0.89 ± 0.10 g), and 38.0% (0.99 ± 0.10 g) at the dose of 10, 5, and 2.5 mg/kg, respectively, while 5-fluorouracil (5-Fu), the positive control, decreased tumor weight by 63.8% (0.58 ± 0.10 g) at the dose of 20 mg/kg, compared with the negative control (1.60 ± 0.13 g). Interestingly, compound **1**, which exhibited stronger toxicity than **2f** both in vitro and in vivo, inhibited tumor growth by equal degree at half-dose of **2f**. In this regard, N-ethylation also resulted in the reduced cytotoxicity in vivo.

The morphological change was also observed in the S180 sarcoma (Fig. 4). In control group, tumor cells grew prosperously with typical characteristics, such as nucleus anachromasis, chromatospherite hypertrophia, and muscle infiltration (Fig. 4A). After treatment of **2f** and **1**, many focal necrosis (NA, see the black arrows, Fig. 4C and D) were found in the sarcoma.

2.6. Survival time in sarcoma 180-bearing mice

The effects of **2f** and **1** on survival time in sarcoma 180-bearing mice were evaluated by measuring the increase of the lifespan. Thus the mouse sarcoma 180 cells were transplanted into Kunming mice by intraperitoneal injection (ip), and the administration of test compounds was started 24 h after inoculation (day 1) and the results are shown in Figure 5. The median survival time (MST) in the control mice was 13.0 ± 1.2 days, while it was increased in a dose-dependent manner by treatment with **2f** and **1**.

Table 2
The effect of **2f** and **1** on tumor growth in mice-bearing sarcoma 180 cells[a]

Group	Dose (mg/kg)	Tumor weight (g)	Inhibitory rate (%)	MTD[b] (mg)
Control	—	1.60 ± 0.13	—	—
5-Fu	20	0.58 ± 0.10	63.8	—
1	1.25	1.03 ± 0.11	35.6	10
	2.5	0.82 ± 0.09	48.8	
	5	0.76 ± 0.06	52.5	
2f	2.5	0.99 ± 0.10	38.0	20
	5	0.89 ± 0.10	44.4	
	10	0.69 ± 0.08	56.9	

[a] Sarcoma 180 cells were implanted subcutaneously into Kunming mice and the tumor weight was measured on the 7th day (ip) after the treatment of chemicals. Data from three separate experiments were given as means ± SD.

[b] Acute maximum tolerated dose (MTD) was defined as the dose just below the lowest dose level that killed at least one female Kunming mouse in a treatment group after a maximum of 15 days.

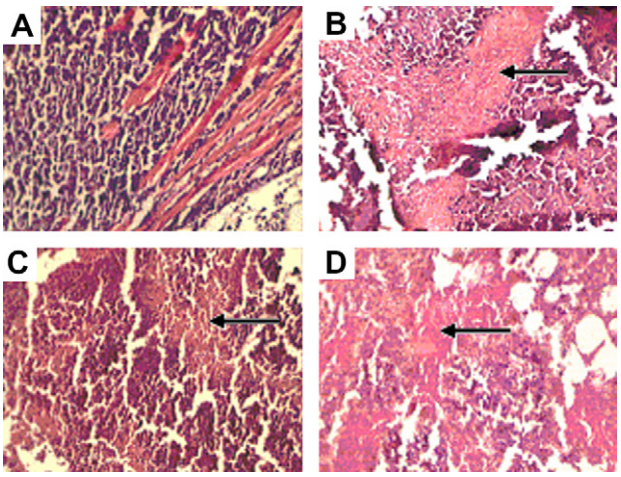


Figure 4. Typical morphological illustration (HE staining; 200×) of the S180 sarcoma. Sarcoma was treated without drug (control, A) or with 20 mg/kg 5-Fu (B), 5 mg/kg **2f** (C), 5 mg/kg **1** (D), respectively.

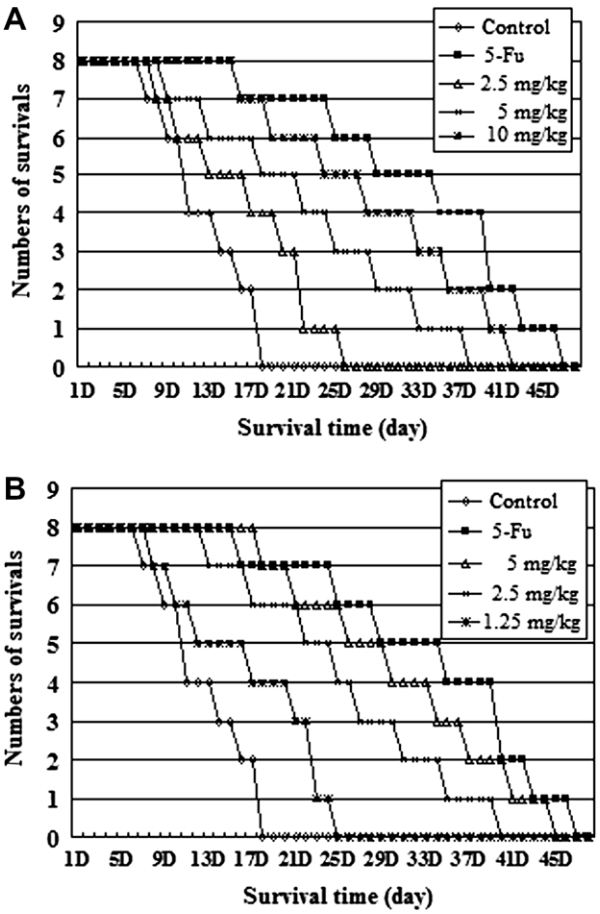


Figure 5. The effect of **2f** and **1** on the increase of the lifespan in mice-bearing sarcoma 180 cells.

After the compound **2f** was ip administrated at the doses of 2.5, 5, 10 mg/kg, the lifespan increased by 1.33 (17.25 ± 1.4 days), 1.80 (23.38 ± 1.9 days), 2.29 (29.75 ± 1.8 days)-fold compared with that of the control group, respectively, while compound **1** increased the lifespan by 1.26 (16.38 ± 1.1 days), 1.94 (25.25 ± 1.4 days), 2.35 (30.5 ± 1.9 days)-fold at the doses of 1.25, 2.5, 5 mg/kg, and 2.64 (34.38 ± 2.1 days)-fold of 5-Fu at the dose of 20 mg/kg, respectively.

3. Conclusion

In summary, a series of unsymmetrically substituted polyamine derivatives were prepared and evaluated for their antitumor activities both in vitro and in vivo. The present survey revealed that the synthesized conjugates were able to recognize the PTS, and the *N*-ethyl modified homospermidine moiety had the potential to be an efficient carrier as homospermidine even though the introduction of *N*-alkyl groups reduced the cytotoxicity in comparison with the un-substituted counterparts. The ODC and TOPO II inhibition experiments demonstrated that there is no direct relationship between ODC and TOPO II inhibition and cytotoxicity of these drugs because compound **1** (the control) was the most cytotoxic in our cell assays and yet the less potent inhibitor in these assays (vs **2f**). This indicated that these enzymes are potential targets, but not unique targets for the antitumor activity of this series. The in vivo evaluation on mice inoculated with sarcoma 180 cells demonstrated that **2f**, as well as **1**, significantly inhibited the tumor growth and increased the survival time. However, it was important to note that the introduction of *N*-alkyl groups also resulted in reduced toxicity in vivo, which might be of clinical benefit. For example, the maximum tolerated dose in mice for **2f** was found to be twice over **1**. Additional studies are required to determine the mechanism by which these polyamine conjugates exert their cytotoxicity, which is an ongoing progress in our laboratory.

4. Experimental

The reagents used were of commercial origin and were employed without further purification. Solvents were purified and dried by standard procedures. Purifications by column chromatography were carried out over silica gel (230–400 mesh). ^{1}H NMR spectra were recorded on a Bruker AV-400 at 400 MHz. Mass spectra were determined by ESI recorded on a Esquire 3000 LC–MS mass instrument. Elemental analyses were performed by the Vario Elemental CHSN-O microanalyzer.

4.1. General procedure for the preparation of *N*-(w-aminoalkyl)-*N*-alkyl-mes-itylenesulfonamides (3a–d)

A solution of mesitylenesulfonyl chloride (10 g, 45.7 mmol) in 100 mL CH_2Cl_2 was added to **2** (41.7 mmol) in 2 N NaOH (100 mL) with ice-bath cooling. The mixture was stirred for 18 h at room temperature, the layers were separated, and the organic layers were washed with 0.5 N HCl (100 mL) and 1:1 H_2O/brine (100 mL) and were concentrated in vacuo. Recrystallization from aqueous EtOH gave the protected alkylamines.

Sodium hydride (60%, 0.99 g, 24.8 mmol) was added to the protected alkylamine (16.5 mmol) in DMF (30 mL) under ice-bath conditions and was stirred for 30 min, then stirring at room temperature for an additional 30 min. *N*-Bromoalkylphthalimide (24.8 mmol) was added followed by heating at 45 °C for 4 h. The reaction was quenched with EtOH (5 mL) and H_2O (10 mL), and solvents were removed in vacuo. The residue was dissolved in $CHCl_3$ and washed with H_2O, dried with anhydrous sodium sulfate, and then the solvent was removed in reduced pressure. Flash chromatography (20% EtOAc/petroleum ether) gave phthalimide intermediates.

A solution of phthalimide intermediate (11.9 g, 27.0 mmol), excess hydrazine hydrate in EtOH were heated at reflux for 12 h, and solvents were removed in vacuo. The residue was dissolved in $CHCl_3$ and washed with H_2O. The organic layer was dried with anhydrous sodium sulfate and concentrated in vacuo. Flash chromatography (10% $CH_3OH/CHCl_3$) afforded **3a–d** as thick oil.

4.1.1. *N*-(3-Aminopropyl)-*N*-ethyl-mesitylenesulfonamide (3a)

Colorless oil (90% yield), ^{1}H NMR (400 MHz, $CDCl_3$, δ): 1.06 (t, J = 7.1 Hz, 3H), 1.33 (m, 2H), 1.59–1.64 (m, 2H), 2.29 (s, 3H), 2.60 (s, 6H), 3.23–3.26 (m, 4H), 6.93 (s, 2H); ESI-MS *m/z*: 285.1 $[M+1]^+$.

4.1.2. *N*-(4-Aminobutyl)-*N*-ethyl-mesitylenesulfonamide (3b)

Colorless oil (100% yield), ^{1}H NMR (400 MHz, $CDCl_3$, δ): 1.06 (t, J = 7.1 Hz, 3H), 1.32–1.36 (m, 4H), 1.51–1.55 (m, 2H), 2.29 (s, 3H), 2.60 (s, 6H), 3.16–3.25 (m, 4H), 6.93 (s, 2H); ESI-MS *m/z*: 299.1 $[M+1]^+$.

4.1.3. *N*-(3-Aminopropyl)-*N*-ethyl-mesitylenesulfonamide (3c)

Colorless oil (85% yield), ^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.77 (t, J = 7.3 Hz, 3H), 1.23–1.26 (m, 2H), 1.45–1.51 (m, 2H), 1.63–1.67 (m, 2H), 2.29 (s, 3H), 2.60 (s, 6H), 3.11 (t, J = 7.6 Hz, 2H), 3.26 (t, J = 7.3 Hz, 2H), 6.93 (s, 2H); ESI-MS *m/z*: 299.1 $[M+1]^+$.

4.1.4. *N*-(4-Aminobutyl)-*N*-propyl-mesitylenesulfonamide (3d)

Colorless oil (100% yield), ^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.78 (t, J = 7.4 Hz, 3H), 1.31–1.36 (m, 4H), 1.47–1.56 (m, 4H), 2.29 (s, 3H), 2.60 (s, 6H), 3.11 (t, J = 7.6 Hz, 2H), 3.17 (t, J = 7.6 Hz, 2H), 6.95 (s, 2H); ESI-MS *m/z*: 313.1 $[M+1]^+$.

4.2. General procedure for the preparation of *N*,*N*′-bis(mesitylenesulfonyl)-*N*-(w-aminoalkyl)-*N*′-alkyl-alkyldiamines (4a–g)

Mesitylenesulfonyl chloride (3.6 g, 16.6 mmol), **3** (15.1 mmol), *N*-bromoalkylphthalimide (15.6 mmol) and hydrazine hydrate (55%, 3.46 g, 59.4 mmol) were reacted according to the procedure for preparation of **3** to afforded **4a–g** as thick oil.

4.2.1. N^1,N^4-Bis(mesitylenesulfonyl)-N^1-(3-aminopropyl)-N^4-ethyl-1,4-butyl-diamine (4a)

^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.99 (t, J = 7.1 Hz, 3H), 1.39–1.40 (m, 4H), 1.64 (m, 2H), 1.80 (br s, 4H), 2.29 (s, 6H), 2.57 (s, 12H), 3.11–3.16 (m, 6H), 3.21 (t, J = 7.1 Hz, 2H), 6.93 (s, 4H); ESI-MS *m/z*: 538.2 $[M+1]^+$.

4.2.2. N^1,N^3-Bis(mesitylenesulfonyl)-N^1-(4-aminobutyl)-N^3-ethyl-1,3-propyl-diamine (4b)

^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.98 (t, J = 7.1 Hz, 3H), 1.26–1.31 (m, 4H), 1.34 (br s, 2H), 1.45 (m, 2H), 1.69–1.70 (m, 2H), 2.29 (s, 6H), 2.55 (s, 12H), 3.03–3.12 (m, 8H), 6.92 (s, 4H); ESI-MS *m/z*: 538.2 $[M+1]^+$.

4.2.3. N^1,N^3-Bis(mesitylenesulfonyl)-N^1-(3-aminopropyl)-N^3-ethyl-1,3-propyl-diamine (4c)

^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.72 (t, J = 7.3 Hz, 3H), 1.47–1.49 (m, 2H), 1.53–1.59 (m, 6H, $4CH_2$+NH_2), 1.69 (m, 2H), 2.29 (s, 6H), 2.55 (s, 12H), 2.96–3.06 (m, 6H), 3.19 (t, J = 7.0 Hz, 2H), 6.93 (s, 4H); ESI-MS *m/z*: 538.2 $[M+1]^+$.

4.2.4. N^1,N^4-Bis(mesitylenesulfonyl)-N^1-(3-aminopropyl)-N^4-propyl-1,4-butyl-diamine (4d)

^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.75 (t, J = 7.4 Hz, 3H), 1.34–1.45 (m, 10H, $8CH_2$+NH_2), 1.58–1.62 (m, 2H), 2.30 (s, 6H), 2.62 (s, 12H), 3.01 (t, J = 7.6 Hz, 2H), 3.12 (m, 4H), 3.19 (t, J = 7.2 Hz, 2H), 6.94 (s, 4H); ESI-MS *m/z*: 552.2 $[M+1]^+$.

4.2.5. N^1,N^3-Bis(mesitylenesulfonyl)-N^1-(4-aminobutyl)-N^3-propyl-1,3-propyl-diamine (4e)

^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.72 (t, J = 7.4 Hz, 3H), 1.28–1.39 (m, 10H, $8CH_2$+NH_2), 1.63–1.75 (m, 2H), 2.28 (s, 6H), 2.55 (s, 12H), 2.95–3.08 (m, 8H), 6.92 (s, 4H); ESI-MS *m/z*: 552.3 $[M+1]^+$.

4.2.6. N^1,N^4-Bis(mesitylenesulfonyl)-N^1-(4-aminobutyl)-N^4-ethyl-1,4-butyldiamine (4f)

^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.96 (t, J = 7.2 Hz, 3H), 1.26–1.31 (m, 2H), 1.36–1.37 (m, 4H), 1.41–1.47 (m, 2H), 1.60 (m, 2H), 2.27 (s, 6H), 2.54 (s, 12H), 3.06–3.14 (m, 8H), 6.90 (s, 4H); ESI-MS m/z: 552.2 $[M+1]^+$.

4.2.7. N^1,N^4-Bis(mesitylenesulfonyl)-N^1-(4-aminobutyl)-N^4-propyl-1,4-butyl-diamine (4g)

^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.71 (t, J = 7.4 Hz, 3H), 1.26–1.35 (m, 2H), 1.36–1.38 (m, 6H), 1.40–1.45 (m, 2H), 1.59 (m, 2H), 2.27 (s, 6H), 2.56 (s, 12H), 2.98 (t, J = 7.6 Hz, 2H), 3.06–3.09 (m, 6H), 6.90 (s, 2H), 6.91 (s, 2H); ESI-MS m/z: 566.3 $[M+1]^+$.

4.3. General procedure for the preparation of *N*-bis(mesitylenesulfonyl)-*N*-(w-anthracen-9-ylmethyl-aminoalkyl)-*N*-alkyl-alkyldiamines (5a–g)

To a stirred solution of amine **4** (10 mmol) in 25% MeOH/CH_2Cl_2 (10 mL) was added a solution of 9-anthraldehyde (10 mmol) in 25% MeOH/CH_2Cl_2 (10 mL) under N_2. The mixture was stirred at room temperature overnight until the imine formation was complete (monitored by TLC). The solvent was evaporated under vacuum to give the crude imine as a bright-green solid.

$NaBH_4$ (30 mmol) was added in small portions to a solution of imine 1:1 CH_3OH/CH_2Cl_2 (20 mL) at 0 °C. The mixture was stirred at room temperature overnight and then concentrated under vacuum. The residue was dissolved in CH_2Cl_2 and washed with aqueous NaOH (0.5 N, 50 mL). The organic layer was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum to afford **5**. Since **5** was unstable, the intermediate was separated by flash column chromatography (5% $CH_3OH/CHCl_3$) and immediately deprotected.

4.4. General procedure for the preparation of the target compounds (2a–g)

Hydrogen bromide in HOAc (48%, 20 mL) was added to **5** (3 mmol) and phenol (10.5 g, 0.112 mol) in CH_2Cl_2 (30 mL) at 0 °C. The mixture was stirred overnight at room temperature and was quenched with H_2O (50 mL). The aqueous layer was separated and washed with CH_2Cl_2 (50 mL), then the aqueous layer was concentrated in vacuo. The residue was treated with 1 N NaOH (10 mL) and 19 N NaOH (5 mL) under ice-bath cooling and was extracted with $CHCl_3$ (50 mL). After concentration of the $CHCl_3$ extracts, the oil was dissolved in EtOH (5 mL) and acidified with concentrated HCl (5 mL) in ice-bath cooling. The precipitate was collected and washed with anhydrous EtOH to give **2** as laurel-green powder.

4.4.1. N^1-3-(Anthracen-9-ylmethylamino)-propyl-N^4-ethyl-butane-1,4-diamine trihydrochloride salt (2a)

Laurel-green powder (36.0% yield), ^{1}H NMR (400 MHz, D_2O, δ): 1.17 (t, J = 7.2 Hz, 3H), 1.64 (m, 4H), 1.99–2.03 (m, 2H), 2.86–3.01 (m, 8H), 3.22 (t, J = 7.9 Hz, 2H), 5.07 (s, 2H), 7.50 (t, J = 7.4 Hz, 2H), 7.61 (t, J = 7.5 Hz, 2H), 8.01 (d, J = 8.1 Hz, 2H), 8.10 (d, J = 8.3 Hz, 2H), 8.50 (s, 1H); ESI-MS m/z: 364.3 $[M+1]^+$; Anal. Calcd for $C_{24}H_{36}Cl_3N_3{\cdot}0.8H_2O$: C, 59.15; H, 7.78; N, 8.62. Found: C, 59.40; H, 7.67; N, 8.61.

4.4.2. N^1-(Anthracen-9-ylmethyl)-N^4-3-(ethylamino)-propyl-butane-1,4-diamine trihydrochloride salt (2b)

Laurel-green powder (39.5% yield), ^{1}H NMR (400 MHz, D_2O, δ): 1.29 (t, J = 7.3 Hz, 3H), 1.71–1.72 (m, 4H), 2.07–2.10 (m, 2H), 3.05 (t, J = 6.8 Hz, 2H), 3.09–3.14 (m, 8H), 4.71 (s, 2H), 7.50 (t, J = 7.4 Hz, 2H), 7.60 (t, J = 7.4 Hz, 2H), 7.86 (d, J = 8.3 Hz, 2H), 7.93 (d, J = 8.8 Hz, 2H), 8.65 (s, 1H); ESI-MS m/z: 364.3 $[M+1]^+$; Anal. Calcd for $C_{24}H_{36}Cl_3N_3{\cdot}0.2H_2O$: C, 60.49; H, 7.70; N, 8.82. Found: C, 60.27; H, 7.61; N, 8.59.

4.4.3. N^1-(Anthracen-9-ylmethyl)-N^3-3-(propylamino)propyl-propane-1,3-diamine trihydrochloride salt (2c)

Laurel-green powder (60.2% yield), ^{1}H NMR (400 MHz, D_2O, δ): 0.88 (t, J = 7.4 Hz, 3H), 1.58–1.63 (m, 2H), 1.95–2.03 (m, 4H), 2.91–3.05 (m, 8H), 3.15 (t, J = 8.0 Hz, 2H), 4.78 (s, 2H), 7.44 (t, J = 7.4 Hz, 2H), 7.54 (t, J = 7.5 Hz, 2H), 7.84 (d, J = 8.3 Hz, 2H), 7.90 (d, J = 7.9, Hz 2H), 8.21 (s, 1H); ESI-MS m/z: 364.3 $[M+1]^+$; Anal. Calcd for $C_{24}H_{36}Cl_3N_3{\cdot}0.3H_2O$: C, 60.26; H, 7.71; N, 8.78. Found: C, 60.33; H, 7.66; N, 8.62.

4.4.4. N^1-3-(Anthracen-9-ylmethylamino)propyl-N^4-propyl-butane-1,4-diamine trihydrochloride salt (2d)

Laurel-green powder (39.2% yield), ^{1}H NMR (400 MHz, D_2O, δ): 0.92 (t, J = 7.4 Hz, 3H), 1.61–1.67 (m, 6H), 2.03–2.05 (m, 2H), 2.95–3.01 (m, 4H), 3.03–3.11 (m, 6H), 4.73 (s, 2H), 7.46 (t, J = 7.4 Hz, 2H), 7.56 (t, J = 7.6 Hz, 2H), 7.87 (d, J = 8.4 Hz, 2H), 7.92 (d, J = 8.8 Hz, 2H), 8.23 (s, 1H); ESI-MS m/z: 378.2 $[M+1]^+$; Anal. Calcd for $C_{25}H_{38}Cl_3N_3{\cdot}1.5H_2O$: C, 58.42; H, 8.04; N, 8.18. Found: C, 58.34; H, 7.71; N, 7.82.

4.4.5. N^1-(Anthracen-9-ylmethyl)-N^4-3-(propylamino)propyl-butane-1,4-diamine trihydrochloride salt (2e)

Laurel-green powder (40.5% yield), ^{1}H NMR (400 MHz, D_2O, δ): 0.90 (t, J = 7.3 Hz, 3H), 1.59–1.62 (m, 2H), 1.95–2.02 (m, 6H), 2.93–3.15 (m, 8H), 3.13–3.16 (m, 2H), 4.75 (s, 2H), 7.49 (t, J = 7.4 Hz, 2H), 7.60 (t, J = 7.4 Hz, 2H), 7.86 (d, J = 8.2 Hz, 2H), 7.90 (d, J = 8.7 Hz, 2H), 8.20 (s, 1H); ESI-MS m/z: 378.3 $[M+1]^+$; Anal. Calcd for $C_{25}H_{38}Cl_3N_3{\cdot}1.4H_2O$: C, 58.63; H, 8.03; N, 8.20. Found: C, 58.92; H, 7.90; N, 7.89.

4.4.6. N^1-(Anthracen-9-ylmethyl)-N^4-4-(ethylamino)butyl-butane-1,4-diamine trihydrochloride salt (2f)

Laurel-green powder (60.2% yield), ^{1}H NMR (400 MHz, D_2O, δ): 1.24 (t, J = 7.3 Hz, 3H), 1.59–1.67 (m, 8H), 3.00–3.06 (m, 8H), 3.16 (t, J = 7.6 Hz, 2H), 5.15 (s, 2H), 7.53 (t, J = 7.5 Hz, 2H), 7.66 (t, J = 7.6 Hz, 2H), 8.03 (d, J = 8.4 Hz, 2H), 8.18 (d, J = 8.8 Hz, 2H), 8.54 (s, 1H); ESI-MS m/z: 378.2 $[M+1]^+$; Anal. Calcd for $C_{25}H_{38}Cl_3N_3{\cdot}1.2H_2O$: C, 59.04; H, 8.01; N, 8.26. Found: C, 59.33; H, 7.89; N, 8.31.

4.4.7. N^1-(Anthracen-9-ylmethyl)-N^4-4-(propylamino)butyl-butane-1,4-diamine trihydrochloride salt (2g)

Laurel-green powder (41.5% yield), ^{1}H NMR (400 MHz, D_2O, δ): 1.07 (t, J = 6.1 Hz, 3H), 1.77–1.85 (m, 10H), 3.09–3.16 (m, 8H), 3.35 (t, J = 6.3 Hz, 2H), 5.17 (s, 2H), 7.69 (t, J = 7.6 Hz, 2H), 7.79 (t, J = 7.4 Hz, 2H), 8.18 (d, J = 8.2 Hz, 2H), 8.26 (d, J = 8.8 Hz, 2H), 8.65 (s, 1H); ESI-MS m/z: 392.4 $[M+1]^+$; Anal. Calcd for $C_{26}H_{40}Cl_3N_3{\cdot}1.4H_2O$: C, 58.63; H, 8.03; N, 8.20. Found: C, 58.94; H, 7.94; N, 8.19.

4.5. Cell culture

The cells of L1210, HeLa and B16, obtained from American Type Culture Collection (ATCC, Rockville, MD, USA), were cultured in RPMI-1640 supplemented with 10% heat-inactivated FCS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin sulfate) at 37 °C in an atmosphere of 95% air and 5% CO_2 under humidified conditions. 1 mmol/L aminoguanidine was added as an inhibitor of amine oxidase derived from FCS and had no effect on the various parameters of the cells measured in this study.

4.6. IC_{50} determinations and methods for transport-related studies

Chemosensitivity was assessed using the thiazolyl blue tetrazolium bromide (MTT) assay. Briefly, 5000 exponentially growing cells (HeLa cells or B16 cells) were seeded onto 96-well, flat-bottomed plates and allowed to attach overnight; 4000 exponentially growing L1210 cells were seeded onto 96-well, flat-bottomed plates. The cells were treated with the increasing concentrations of synthetic polyamine conjugates in the absence and the presence of DFMO (5 mM) or SPD (500 μM) for 48 h, and 100 μL MTT (1 mg/mL) was added to each well. After incubation at 37 °C for 4 h, the MTT solution was removed and the crystals of the viable cells were dissolved with DMSO (HeLa cells or B16 cells) or 10% SDS (L1210 cells). The absorbance of each well was read at 570 nm. The inhibition rate was calculated from plotted results using untreated cells as 100%.

4.7. ODC assays

ODC activity was measured by the released $^{14}CO_2$ from labeled ornithine. ODC 4293 cells were cultured in a six-well plate at a density of 1×10^5 per well in DMEM (Cellgro) supplied with 10% (Invitrogen), Charcaol/Dextran-treated FBS (Hyclone), 200 ng/mL G418 (Calbiochem), 200 ng/mL Zeocin, and 5 μM Ponasterone A (Invitrogen) for 24 h. The conjugates tested were then added into the cells at 5 μM for another 24 h. Sample collection and the assay procedure were carried out as described previously.[25]

4.8. Topoisomerases assays[28]

The decatenation assays were performed in buffer A containing 50 mM Tris–HCl (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 30 μg/mL bovine serum albumin, 0.5 mM dithiothreitol, and 0.5 mM ATP. Reaction mixtures contained 0.25 μg of catenated kinetoplast DNA, 1.5 U of human topoiosomerase II, and the appropriate polyamine conjugate (**1**, **2f**) at 5 μM, 10 μM, 20 μM, respectively (Fig. 3). Controls were run in parallel. Reaction mixtures were incubated at 37 °C for 30 min. Decatenation reactions were quenched with 20 mM Na_2EDTA and 100 μg/mL proteinase K. Reaction products were separated by electrophoresis at 2.5 V/cm through a 1% agarose gel. The gel contained 0.5 μg/mL ethidium bromide and was submerged in buffer B [1 × TAE buffer: 40 mM Tris–HCl, 25 mM sodium acetate, and 1 mM EDTA (pH 8.5)]. The reaction products were visualized under ultraviolet light and photographed.

4.9. In vivo testing of maximum tolerated dose

The acute maximum tolerated dose (MTD) was determined following single administration by intraperitoneal injection of increasing drug doses to groups of three healthy female Kunming mice. Seven dose levels of each drug (0.625, 1.25, 2.5, 5, 10, 20, 25 mg/kg) were evaluated. The MTD was defined as the dose just below the lowest dose level that killed at least one mouse in a treatment group after a maximum of 15 days.

4.10. Tumor growth inhibition in mice inoculated sarcoma 180 cells[29]

Female Kunming mice were purchased from laboratorial animal center of Henan (Henan, China). All animal studies were carried out in a pathogen-free barrier zone at Henan University in accordance with the procedure outlined in the Guide for Care and Use of Laboratory animal. Animals were fed on sterilized animal chow and water ad libitum and they were housed at 23 ± 0.5 °C, 10% humidity under a 12-h light–dark cycle. The effect of drug on tumor growth was measured by evaluating tumor weight. For solid tumor development, the Kunming mice (6–8 weeks olds, weighing 18–20 g) were injected subcutaneously with 2×10^6 of sarcoma 180 cells. The next day after inoculation, mice randomized into eight groups were administrated by intraperitoneal injection with **2f** (10, 5 and 2.5 mg/kg), **1** (5, 2.5 and 1.25 mg/kg), physiologic saline or 5-Fu (20 mg/kg) for consecutive 7 days. On day 8, the mice were killed by cervical dissociation, and solid tumors were removed and weighed. The inhibitory rate was calculated as follows: inhibitory rate (%) = $[(A - B)/A] \times 100$, where A was the mean tumor weight of the negative control group, and *B* was that of the drug treated or positive group.

4.11. Enhancement of survival time in mice-bearing sarcoma 180 cells

For calculating the survival time, Kunming mice were inoculated ip with 1×10^6 sarcoma 180 cells/mouse on day 0 and the treatment with three doses of **2f** (10, 5 and 2.5 mg/kg, ip) or **1** (5, 2.5 and 1.25 mg/kg, ip) was started 24 h after inoculation for 8 consecutive days. The control group was treated with physiologic saline. The median survival time (MST) for each group was observed and the antitumor activity of drug was evaluated by measuring the increase of the lifespan.

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