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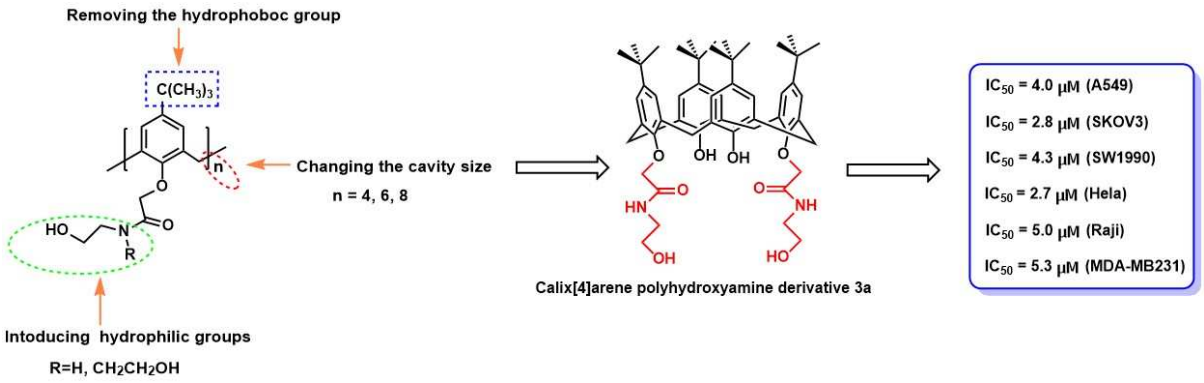

Synthesis, X-ray crystal structure and anti-tumor activity of calix[n]arene polyhydroxyamine derivatives

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a b s t r a c t

Calixarene-based compounds are highly effective therapeutic agents against cancer. This study aims to prepare a series of calix[n]arene (n = 4, 6, 8) polyhydroxyamine derivatives (**3a–3m**) and to study their potential antitumor activities. The single crystal structure of calix[4]arene derivative **3a** was determined through X-ray diffraction. We assessed the ability of the prepared calix[n]arene polyhydroxyamine derivatives to induce cytotoxicity in six cancer cell lines by performing cancer cell growth inhibition assays. Results demonstrated that compounds **3a–3d** achieved IC_{50} values ranging from 1.6 μM to 11.3 μM. Among the different compounds, **3a** and **3b** exerted the strongest cytotoxic effect in inhibiting the growth of SKOV3 cells. In relation to the underlying mechanisms of cytotoxic effects, cell cycle analysis revealed that the exposure of SKOV3 cells to **3a** induced cell cycle arrest in the G0/G1 phase, suggesting a reduction in DNA synthesis. Immunofluorescent staining indicated that the protein expression levels of caspase-3 and p53 in cells significantly increased, whereas that of Bcl-2 was effectively suppressed. Meanwhile, no significant changes in Bax were observed in SKOV3 cells. These results highlight that calixarene **3a** can be further studied as a potential anticancer agent.

*Keywords:*calix[n]arene, polyhydroxyamine derivative, anti-tumor activity.

1. Introduction

In the past decades, calix[n]arenes comprising phenol units bridged by methylene linkers have been proven to be an important family of host molecules in supramolecular chemistry. Moreover, calix[n]arenes could be functionalized at the upper and lower rims and readily converted into various derivatives such as esters, carboxylates, ethers, and amides [1-3]. In this case, calix[n]arene and their derivatives exhibit molecular recognition of ions [4, 5], neutral molecules [6], peptides [7], amino acids [8], proteins [9], and nucleic acids[10], due to their flexible macrocyclic backbones. Recently, their potential applications in biomedical fields [11-12] as enzymatic inhibitors [13-16], antimicrobials[17-19], and antitumor agents [20-26] have gained considerable attention. To the best of our knowledge, water-soluble calixarenes modified with anionic sulfonate, phosphate, carboxylate, or cationic tetra-alkyl ammonium functional groups could solubilize [27, 28]and deliver drugs [29, 30]. Recent studies have found that *p*-sulfonato-calix[6]arene and *p*-sulfonato-calix[8]arene exert antitumor effects on leukemia cell lines[31]. In addition, neutral, hydrophilic substituents has been proposed as an alternative means of rendering calixarenes water-soluble[32]. Research on neutral water-soluble calixarene-attaching glycosylated or polyhydroxylated groups have been investigated both in synthesis and biological evaluation[23, 33-37]. From this perspective, searching for new functionalized calixarenes as antitumor agents and exploring the molecular mechanisms underlying their actions are ongoing challenges. The present work aimed to report the convenient synthesis of calix[n]arene (n = 4, 6, 8) polyhydroxyamine derivatives **3a–3m** and assess the ability of **3a–3d** (**Fig.1**) to induce cytotoxicity in six cancer cell lines, namely, the human lung cancer cell line A549, the ovarian cancer cell line SKOV3, the pancreatic cancer cell line SW1990, the cervical cancer cell line Hela, Burkitt's lymphoma cell line Raji, and the breast cancer cell line MDA-MB-231. The mechanisms underlying the potential antitumor properties of these compounds were also elucidated.

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+L. L. Han contributed equally to this work.

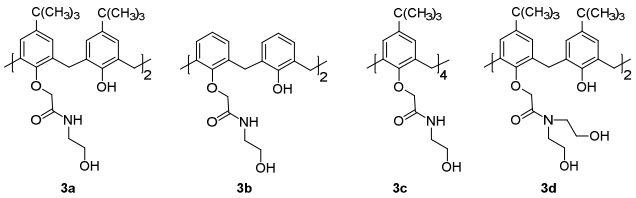


Figure1. Structural representations of calix[4]arene polyhydroxyamine derivatives **3a–3d**

2. Results and discussion

2.1 Rational anti-tumor agents design based on calixarenes

Recent years, studies on anti-tumor activity of various functionalized calixarene derivatives have been very modestly investigated. Dings *et al*[22, 24-25] reported some calix[4]arene amides were found to be highly effective at inhibiting various tumor cells. Pelizzaro-Rocha *et al* [26] revealed that calix[6] arene was potent in reducing Panc-1 cell viability. Thus, the amino or acylamino groups at the lower rim of calix[n]arene are essential for inhibitory activity. Likewise, alcohols or hydramine are by far the most used functional groups to improve the physicochemical property of anti-tumor agents. With the goal of developing novel and effective anti-tumor agents based on calixarenes, we used the *tert*-butyl calix[4]arene as a model core to design a series of calixarene polyhydroxyamine derivatives via changing the cavity size, removing the hydrophobic *tert*-butyl group at the upper rim and introducing hydrophilic polyhydroxyamine group at the lower rim (**Fig.2**).

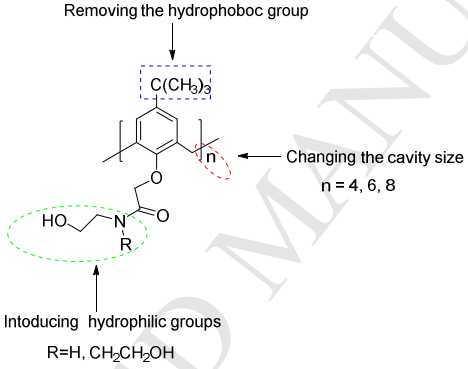
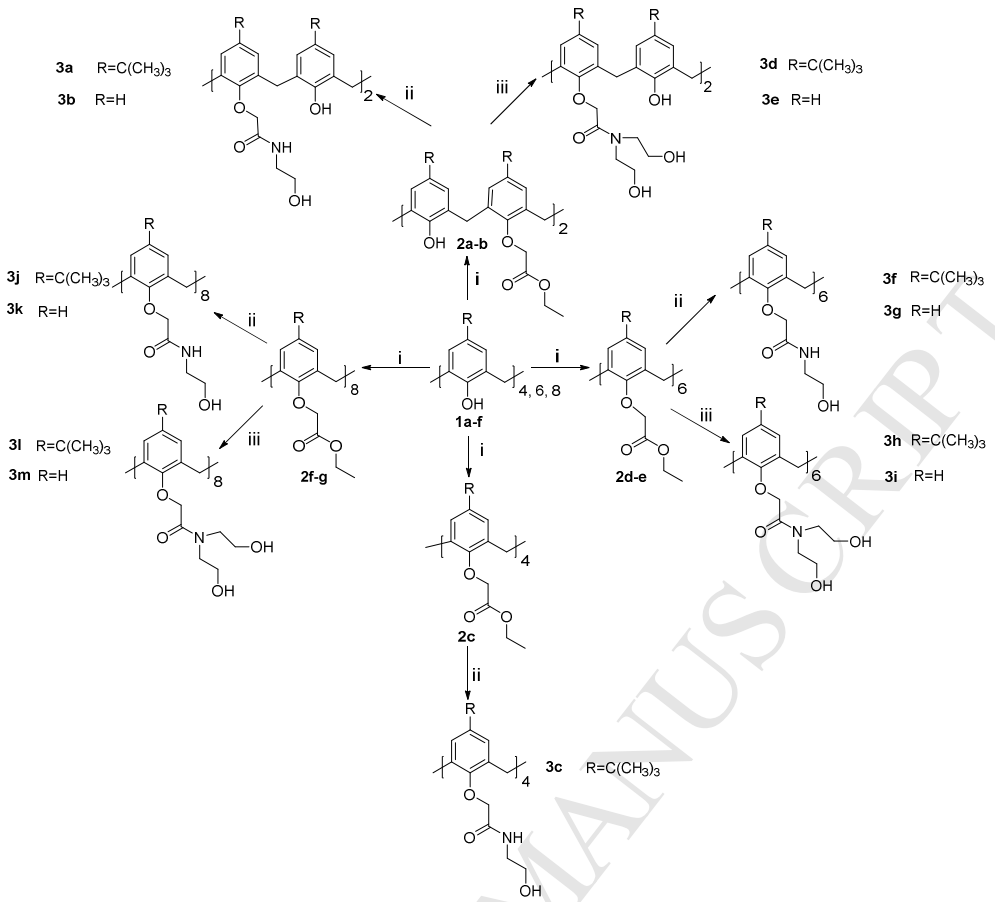


Figure2. Structural optimization design of calix[n]arene

*2.2 Synthesis of the polyhydroxyamine calix[n]arene derivatives **3a–3m***

A convenient procedure for the synthesis of polyhydroxyamine calix[n]arene derivatives **3a–3m** is shown in **Scheme 1**. Calix[n]arene polyhydroxyamine derivatives can be synthesized through the direct ammonolysis reaction of calixarene esters with hydroxyamines [2] or through the condensation reaction between calixarene carboxylic acids and hydroxyamines [38]. Considering that ethanolamine and diethanolamine possess less steric hindrance, we selected the first synthetic method. As shown in Scheme **1**, the desired calixarene ester derivatives were prepared by treating p-tert-butylcalix[n]arenes **1a-c** (n = 4, 6, 8) and calix[n]arenes **1d–1f** (n = 4, 6, 8) with ethyl chloroacetate in the presence of anhydrous K_2CO_3 and KI in refluxing acetone. Ultrasonic irradiation was introduced into this reaction to reduce the reaction time from 3–5 days to approximately 12 h. To our satisfaction, seven corresponding calix[n]arene ester derivatives (**2a–2g**) were obtained in 75%–85% yields. Subsequently, calixarene esters **2a–2g** reacted with excess ethanolamine or diethanolamine in a refluxing solvent of 1:1 EtOH/toluene to produce the desired calixarene polyhydroxyamine derivatives **3a–3m** in moderate yields from 25% to 84%. Ethanolamine and diethanolamine reagents were chosen because they were commercially available, inexpensive, and highly water soluble. Calix[8]arene polyhydroxyamines **3l–3m** were obtained in lower yields (25%, 27%) possibly because of the steric congestion of eight diethanolamine scaffolds at the lower rim of calix[8]arene. By contrast, calix[4]arene 1,3-disubstituted hydroxyamine derivatives were successfully prepared in high yields by using calix[4]arene 1,3-diesters as the starting material. The unreacted hydroxyamines in the reaction were separated from the calixarene polyhydroxyamine derivatives by washing the extracted crude material with water/chloroform. Further product purification can be achieved by performing column chromatography with silica as the solid support.



Scheme1. Synthesis of calix[n]arene polyhydroxyamine derivatives **3a–3m**
Reagents and conditions: (i) $ClCH_2COOC_2H_5$, K_2CO_3, acetone, ultrasonic irradiation; (ii) $NH_2CH_2CH_2OH$, ethanol/toluene,reflux; (iii) $NH(CH_2CH_2OH)_2$, ethanol/toluene, reflux.

*2.3 X-ray crystal structure of **3a***

The single crystal structure of derivative **3a** is shown in **Fig. 3** and the crystal data and refinements are listed in **Table 1**.

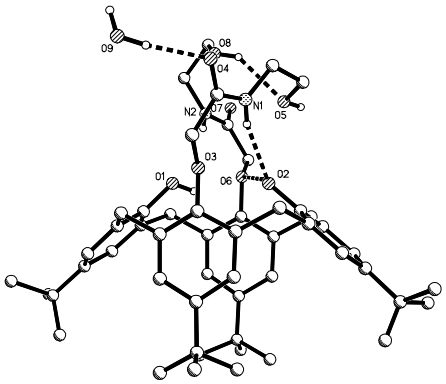


Figure 3. Single crystal structure of **3a** (hydrogen atoms on the skeletons were omitted for clarity)

It is clearly seen that calix[4]arene core adopts a cone-conformation. The two N-(2-hydroxyethyl) -acetamide chains exist in 1,3-distal position. In molecules, the two p-t-butylphenol units bearing with acetamide chain stand more perpendicular (dihedral angle: 82.122(170)°, 89.500(18)°) to the other two *p*-tert-butylphenol units without substituents (dihedral angle: 40.905(133)°, 41.384(146)°). There are several intramolecular and intermolecular hydrogen bonds formed by the interaction of the phenolic hydroxy group and N-(2-hydroxyethyl)-acetamide chain.

Table 1. Crystal data for Compound **3a**

Phase	**3a**
Empirical formula	$C_{52}H_{72}N_2O_9$
Formula weight	869.12
Temperature	296(2)
Wavelength/nm	0.71073
Crystal system	Triclinic
space group	P -1
a (Å)	9.937(4)
b (Å)	12.228(4)
c (Å)	22.273(6)
α(°)	87.276(10)
β(°)	82.963(13)
γ(°)	72.794(13)
Volume(Å3)	2565.7(14)
Z	2
F(000)	940
Crystal size (mm)	0.36 x 0.34 x 0.28
Calculated density (g.cm-3)	1.125
Absorption coefficient (mm-1)	0.076
θ range (°)	0.92 to 25.00
Limiting indices	-11 < =h < =11, -14 < =k < =14, -26 < =l < =26
Reflections collected / unique	29477 / 8702 [R(int.) = 0.1267]
Completeness to theta	96.3 %
Max. and min. transmission	0.979 and 0.973
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	8702 / 72 / 601
Goodness-of-fit on F^2	1.038
Final R indices [I > 2sigma(I)]	R_1 = 0.1203, wR_2 = 0.2627
R indices (all data)	R_1 = 0.2326, wR_2 = 0.3168
Largest diff. peak and hole(e.Å-3)	0.589 and -0.456

*2.4 Compounds **3a–3m** reduce the viability of six differenet cancer cells*

The efficiencies of the calix[n]arene polyhydroxyamine derivatives **3a–3m** as anti-cancer drugs and tools for biological research were examined. We first evaluated their ability to induce cytotoxicity through preliminary screening in six cell lines, namely, A549, SKOV3, SW1990, Hela, Raji and MDA-MB-231. Cell viability was assessed 72 h after treatment by conducting an MTT or SRB assay, and preliminary screening results are shown in **Table 2**. In general, the different cavities and molecular footprints of the calixarene scaffold showed different results. Calix[4]arene derivatives **3a–3e**, each of which contains four phenolic units, showed better inhibitory effects on the cell lines than the other derivatives. By contrast, calix[6]arenes and calix[8]arenes **3f–3m** possessing six or eight phenolic units showed poor or even no viability. We assume that large molecular structures cannot easily permeate the cell membrane. These results indicated that the cavity size of calix[n]arene was a critical factor for its efficiency against cancer cells. The absence of activity of **3f–3m** illustrated that enlarging the cavity of the calixarenes greatly attenuated the activity. Interestingly, the introduction of a hydrophobic group (e.g., *tert*-butyl groups) at the upper rim and a short-chained hydrophilic face at the lower rim of calixarenes improved the activity of these compounds regardless of calixarene cavity size. For example, **3a** and **3c** bearing two or four O-acetyl ethanolamine groups at the lower rim

were effective at inducing cytotoxicity in various cell types; these groups were tested and showed almost 100% single concentration inhibition. However, **3e** showed a low inhibitory effect on the cancer cell types because of the removal of *tert*-butyl groups and the introduction of long-chained diethanolamine groups.

Table 2. Single concentration inhibition of calixarenes **3a–3m** on cancer cell viability

Compound	Single concentration inhibition (%)[a]					
	A549	**SKOV3**	**SW1990**	**Hela**	**Raji**	**MDA-MB-231**
3a	89	97	95	100	100	99
3b	13	86	10	46	29	57
3c	93	95	99	100	100	99
3d	16	42	59	95	77	95
3e	7	34	4	38	34	22
3f	7	24	4	40	38	12
3g	7	10	3	6	18	4
3h	0	26	26	0	14	0
3i	5	19	0	9	6	22
3j	8	26	5	23	24	12
3k	2	1	4	32	7	27
3l	0	25	5	10	11	11
3m	6	25	2	18	11	13

[a] the concentration of the calixarenes **3a–3m** was 10 μM

Basing from the above results, we treated with **3a–3d**. Dose response curves are shown in **Fig. 4** as the percentage of cell survival relative to that from cultures treated only with the vehicle (100%). Data points represented the means obtained in at least three independent experiments, each using triplicate cell cultures. Apparent IC_{50} values (i.e., the concentration required for 50% cell viability) derived from these curves are given in **Table 3**.

Table3. IC_{50} values (μM) of calixarenes **3a–3d** on various cancer cells

Cancer cells	IC_{50} (μM)			
	3a	**3b**	**3c**	**3d**
A549	4.0	--	3.7	--
SKOV3	2.8	2.3	5.1	--
SW1990	4.3	--	3.3	11.3
Hela	2.7	--	7.1	8.1
Raji	5.0	--	4.7	11.2
MDA-MB231	5.3	1.6	3.4	5.2

"--"means no detected

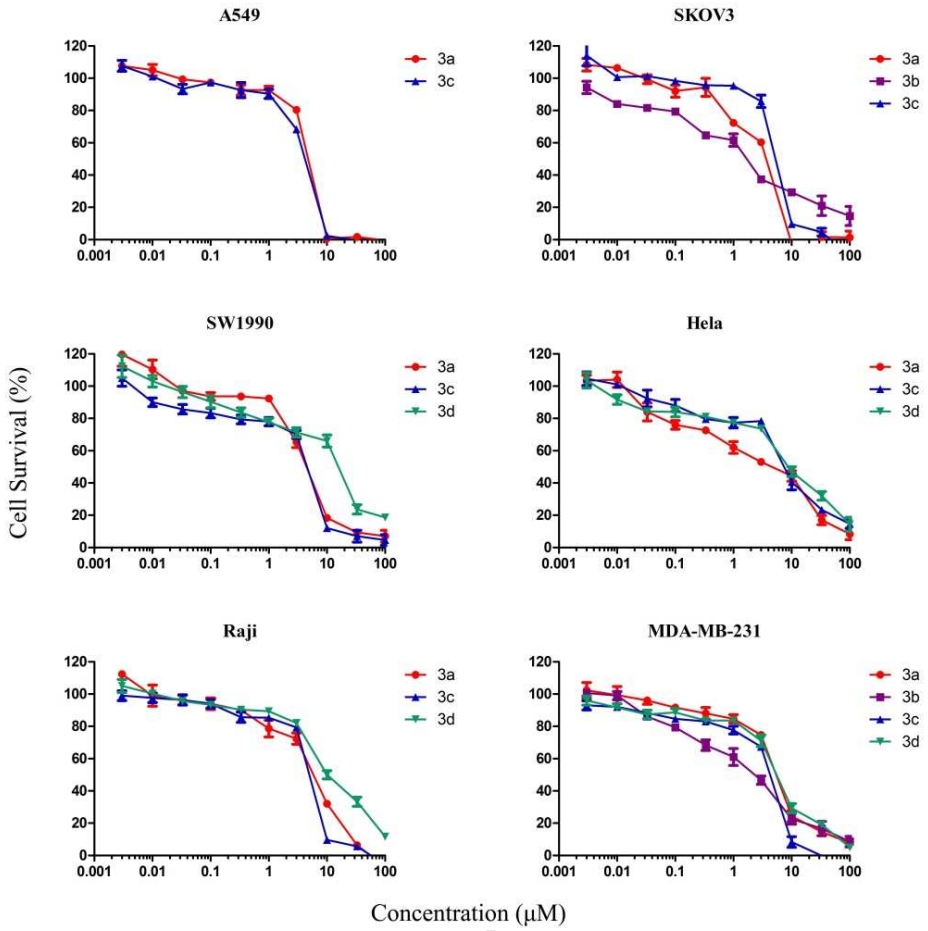


Figure 4. Cytotoxicity effects of calix[4]arenes **3a–3d**. Cell viability of A549, SKOV3, SW1990, Hela, Raji as well as MDA-MB231cells was measured in the presence of various concentrations of calixarenes.

Compounds **3a–3d** achieved IC_{50} values ranging from 1.6 μM to 11.3 μM. Importantly, **3a** and **3c** exerted great cytotoxic effects in inhibiting the growth of the six types tested. Compound **3a** showed a narrow activity variation from 2.7 μM to 5.3 μM (the single digit micromolar activity is approximately 4 μM) against all the tested cell types. These results revealed that the tert-butyl groups introduced by calix[4]arene into the upper rim significantly increased the cytotoxic function of the compounds. By contrast, **3b** was only selectively effective against the SKOV3 and MDA-MB231 cells (IC_{50} of 2.3 μM against SKOV3 and 1.6 μM against MDA-MB231). Meanwhile, **3d** showed a reduced potency over the cytotoxicity profiles of **3a** and **3c**.

Compounds **3a** and **3b** were more effective than the other calix[n]arenes in inhibiting the growth of SKOV3. Chemical antitumor agents can effectively inhibit cell proliferation by inducing cell cycle arrest [25, 26]. To clarify how calix[n]arenes suppress cell growth, cell cycle progression was analyzed by quantitating DNA content through flow cytometry in SKOV3 cells [39]. We treated SKOV3 cells with different concentrations (0, 0.3, 1, 3.3, and 10 μM) of calixarene derivatives **3a** and **3b** after 24 and 48 h, respectively, and measured DNA content through propidium iodide staining (**Fig. 5**).

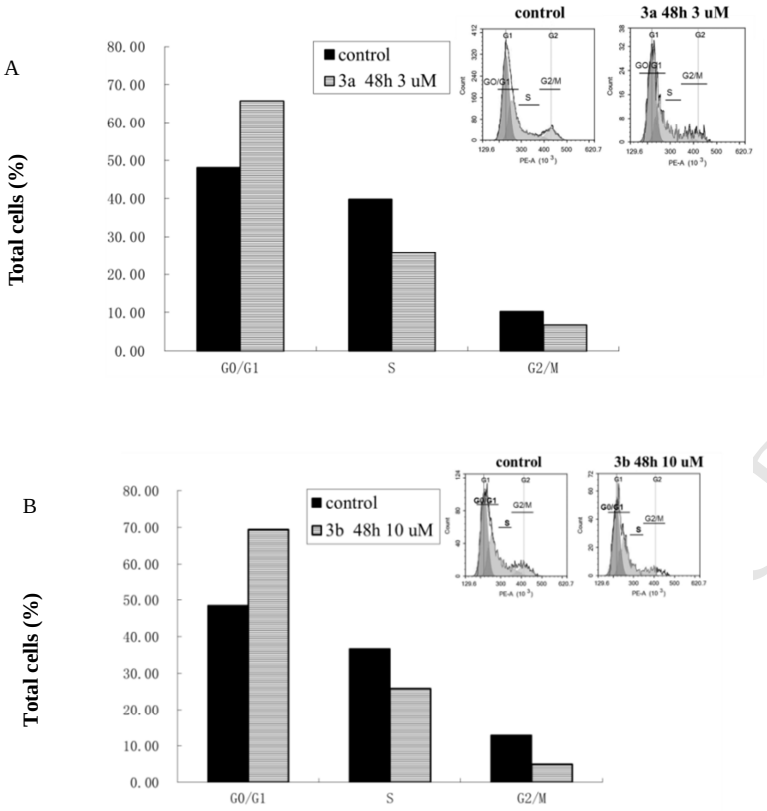


Figure 5. The amount of SKOV3 cells each cell cycle phase (G0/G1 phase, S phase and G2/M) after treatment with specified concentrations of **3a** (A) and **3b** (B) for 48 h was determined by propidium iodide staining followed by flow cytometry.

As shown in Fig. 5A, 64.25% ± 2.26% of cells treated with a low concentration of **3a** (3 μM) were arrested in the G0/G1 phase compared with the 49.47% ± 1.91% of the nontreated cells. Accordingly, the percentage of cells in the S phase reduced from 37.31% ± 3.48% in the nontreated cells to 24.19% ± 2.26% in the cells treated with 3.0 μM **3a**. The same profile was observed in the cells treated with 10 μM **3b** (Fig. 5B). Moreover, cell cycle analysis revealed that **3a** and **3b** induced cell cycle arrest in the G0/G1 phase. This result was supported by the increased cell count in the G0/G1 phase at 3 and 10 μM for 48 h and the decreased cell count in the S and G2/M phases.

2.5 Immunofluorescent staining study of 3a

To further illuminate the molecular of **3a** mediated apoptosis in SKOV3 cells, immunofluorescent staining was used to evaluate apoptotic related proteins Caspase-3, P53, Bcl-2, and Bax protein levels[40, 41]. As shown in **Fig.6**, after SKOV3 cells were treated with certain concentration (3μM)of calixarene derivative **3a** for 48h, Caspase-3, p53 protein levels in SKOV3 cells were upregulated significantly, whereas that of Bcl-2 protein was obviously downregulated, meanwhile no significant changes of Baxprotein was observed in SKOV3 cells, compared to DMSO group. Therefore, these results suggested that **3a** might induce cellular apoptosis through these proteins in SKOV3 cells.

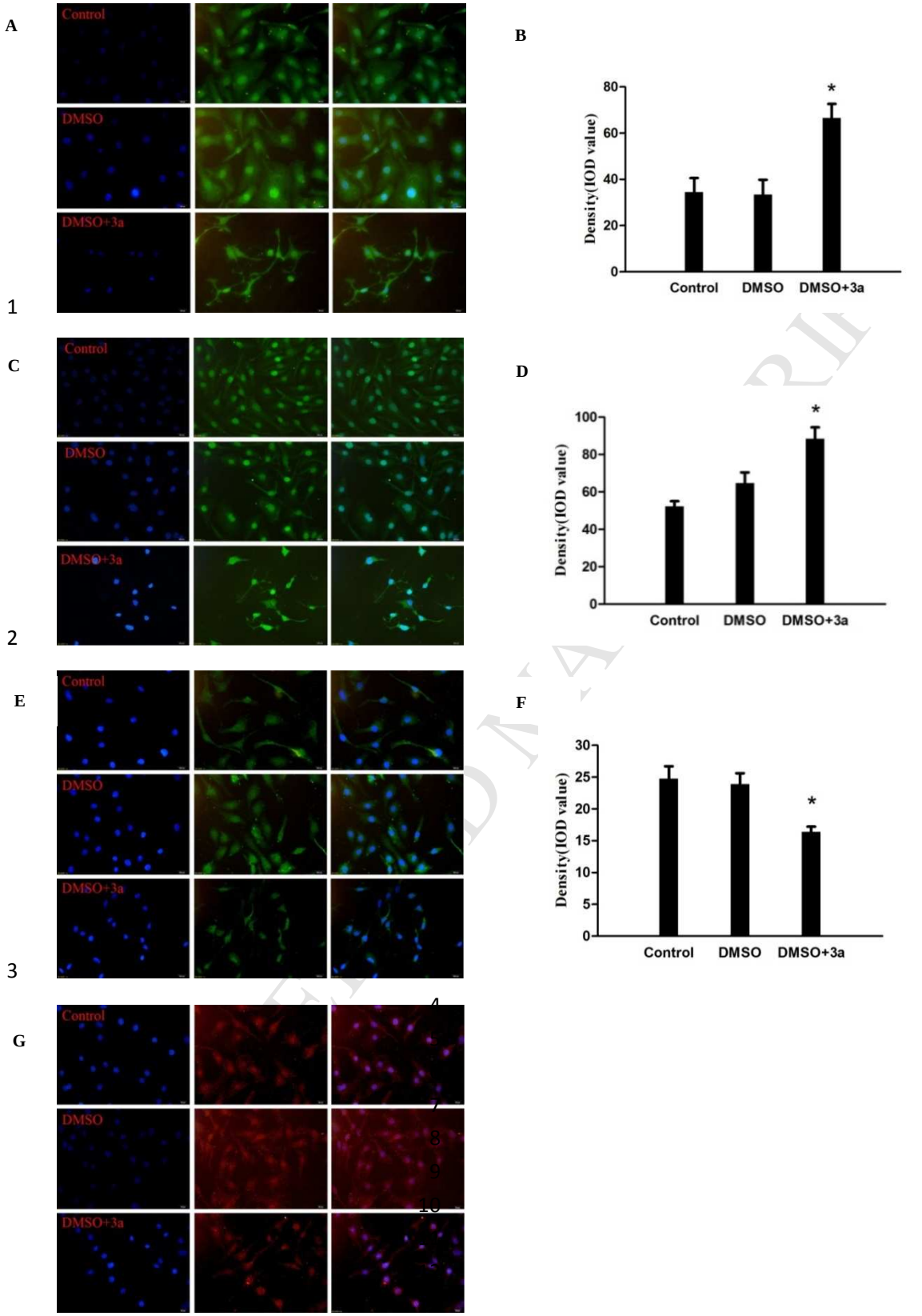


Fig.**6** Effects of apoptotic related proteins expression on the apoptosis of treated and untreated SKOV3 cells. Caspase-3 (A), P53 (C), Bcl-2 (E), Bax (F) protein antibody and fluorescein-conjugated second antibody were used to visualize corresponding protein, and Hoechst 33342 was used to stain cell nuclei (blue).The levels of Caspase-3 (B), P53 (D) are increased in SKOV3 cells, the level of Bcl-2 (E) is decreased with Bax protein no changes.

3. Conclusion

The results presented in this study showed that calix[4]arene polyhydroxyamine derivatives **3a** are highly effective in inducing cell death in human ovarian carcinoma cells. Our findings provide strong evidence of the large effects of **3a** in reducing SKOV3 cell aggressiveness. We also find that **3a** could result in apoptotic cell death by up-regulation of Caspase-3, p53 proteins In SKOV3 cells with keeping Bax no change. Overall, calixarene **3a** can be further studied as a potential anticancer agent in the future.

4. Experimental section

4.1. Chemistry synthesis

p-tert-butylcalix[n]arenes **1a–1c** (n = 4, 6, 8) were synthesized according to Gutsche's method [42]. Calix[n]arenes **1d–1f** (n = 4, 6, 8) were obtained by deakylation of calixarenes **1a–1c** with $AlCl_3$ in toluene [43]. calix[n]arenes esters **2a–2g** were prepared by treating **1a–1f** with chloroacetate in the presence of K_2CO_3/KI/acetone under ultrasonic irradiation[44]. Other reagents and solvents were commercial reagents with analytical grade and utilized without further purification. All compounds were dissolved in $CDCl_3$, or DMSO-d_6 and the ^1H and ^{13}C NMR spectra were recorded at 400 MHz on a Bruker AV-400 spectrometer or on a JEOL JNM-ECS400 spectrometer. The chemical shifts were reported as δ values in parts per million (ppm) and coupling constants (J) were given in hertz (Hz). The peak pattern abbreviations are as follows: s, singlet; brs, broad singlet, d, doublet; q, quadruplet; dd, doublet of doublet; t, triplet; m, multiplet. IR spectra were obtained on a Nicolet FT-IR 8400 spectrometer (KBr disc). Mass spectra were carried out on (UHR-TOF) maXis 4G mass spectrometers. Purification was performed by using preparative separations in flash column chromatography. Melting points were determined with capillaries with an YRT-3 microscope apparatus and were uncorrected. Reactions were monitored by thin layer chromatography (TLC) on 2.5 mm Merck silica gel F254 strips.

*4.1.1 General procedure for the synthesis of 5,11,17,23-tetra-tert -butyl-25,27-bis [N-(2-hydroxyethyl) aminocarbonylmethoxyl] calix[4]arene-26,28-diol (**3a**)*

A mixture of calix[4]arene ester **2a** (820mg, 1.0 mmol) and ethanolamine (244mg, 4.0mmol) was dissolved in refluxing ethanol/toluene (50 mL, v/v = 1:1) for 24h until complete by TLC. The solution was extracted with water/chloroform. The combined chloroform layers were washed with brine solution, dried over $MgSO_4$ and concentrated invacuo. Then, the oil residue was dissolved in 95% ethanol and the resulting precipitate was collected by filtration, which was purified by column chromatography (SiO_2, 1:3 petroleum ether/ethyl acetate to give product **3a** (637 mg, 75%) as a white solid. Mp =244.6-247.9℃, ^1H NMR ($CDCl_3$, 400 MHz) δ: 1.01, 1.29 (2s, 36H), 3.42 (d, *J* = 13.2 Hz, 4H), 3.59 (q, *J* = 4.8 Hz, 4H), 3.81 (t, *J* = 4.8 Hz, 4H), 4.18 (d, *J* = 13.2 Hz, 4H), 4.58 (s, 4H), 6.89 (s, 4H), 7.10 (s, 4H), 7.60 (brs, 2H), 9.11 (t, *J* = 5.2 Hz, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 30.9, 31.6, 32.0, 33.9, 34.1, 42.4, 61.7, 74.8, 125.6, 126.2, 127.5, 132.3, 143.3, 148.3, 149.3, 149.4, 169.8; IR (KBr) υ: 3385, 3338, 2961, 2868, 1666, 1558, 1486, 1452, 1363, 872 cm^{-1}; MS m/z: HRMS (ESI) Calcd: for $C_{52}H_{70}N_2NaO_8$ ($[M+Na]^+$): 873.5024, found: 873.5066.

25, 27-bis-[N-(2-hydroxyethyl)aminocarbonylmethoxyl]-calix[4]arene-26,28-diol (**3b**): White powder, purified by column chromatography (SiO_2, 1:3 petroleum ether/ethyl acetate), Mp > 250℃, yield: 84 %; ^1H NMR (DMSO-d_6, 400MHz) δ: 3.43 (q, *J* = 4.0 Hz, 4H), 3.47 (d, *J* = 8.8 Hz, 4H), 3.56 (brs, 4H), 4.23 (d, *J* = 8.8 Hz, 4H), 4.53 (s, 4H), 4.72 (brs, 2H), 6.62 (t, *J* = 5.2 Hz, 2H), 6.81 (t, *J* = 5.2 Hz, 2H), 7.06 (d, *J* = 4.8 Hz, 4H), 7.17 (d, *J* = 4.8Hz, 4H), 8.30 (s, 2H), 8.54 (t, *J* = 3.6 Hz, 2H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 31.0, 41.8, 60.2, 60.3, 61.6, 74.7, 119.8, 119.9, 120.1, 126.1, 127.8, 127.9, 128.1, 128.2, 129.1, 129.2, 129.4, 129.5, 129.6, 129.8, 133.9, 134.0, 134.1, 134.3, 134.4, 152.5, 152.6, 152.7, 152.8, 167.4, 168.4; IR (KBr) υ: 3337, 3318, 2925, 1682, 1667, 1545, 1464, 1439 1343, 1250, 764 cm^{-1}; MS m/z: HRMS (ESI) Calcd: for $C_{36}H_{38}N_2NaO_8$ ($[M+Na]^+$): 649.2520, found: 649.2548.

5,11,17,23-tetra-*tert*-butyl-25,26,27,28-tetra[N-(2-hydroxyethyl)aminocarbonylmethoxyl]-calix[4]arene (**3c**): White powder, purified by column chromatography (SiO_2, 10:1 ethyl acetate/methanol), Mp =195.8-197.1℃, yield: 67 %; ^1H NMR ($CDCl_3$, 400 MHz) δ: 0.83 (s, 18H), 1.33, 1.34 (2s, 18H), 3.34 (q, *J* = 6.8 Hz, 5H), 3.48-3.57 (m, 5H), 3.66 (q, *J* = 4.8 Hz, 2H) 3.72-3.81 (m, 5H), 3.86 (t, *J* = 4.8 Hz, 2H), 4.27 (q, *J* = 6.4 Hz, 5H), 4.38 (dd, *J* = 10.0 Hz, 5H), 4.56 (t, *J* = 16.4 Hz, 3H), 5.71 (s, 1H), 6.52 (d,

J = 2.0 Hz, 2H), 6.58 (d, *J* = 2.0Hz, 2H), 7.10 (s, 2H), 7.19 (s, 2H), 7.64 (t, *J* = 5.6 Hz, 2H), 8.39 (t, *J* = 5.6 Hz, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 30.9, 31.0, 31.2, 31.4, 31.6, 31.7, 33.8, 34.0, 34.3, 42.3, 42.4, 61.5, 75.5, 125.6, 126.6, 129.0, 131.1, 131.3, 135.0, 146.8, 147.6, 149.3, 151.0, 169.0, 171.0; IR (KBr) υ: 3381, 2961, 2868, 1659, 1555, 1481, 1194, 1124, 872 cm^{-1}; MS m/z: HRMS (ESI) Calcd: for $C_{60}H_{84}N_4NaO_{12}$ ([M+Na]$^+$): 1075.5983, found: 1075.6011.

5,11,17,23-tetra-*tert*-butyl-25,27-bis[N,N-di(2-hydroxyethyl)aminocarbonylmethoxyl]-calix[4]arene-26,28-diol (**3d**): White powder, purified by column chromatography (SiO_2, 1:3 petroleum ether/ethyl acetate), Mp > 250℃, yield: 81%; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 1.05, 1.19 (2s, 36H), 3.50 (q, *J* = 5.2 Hz, 8H), 3.62 (brs, 8H), 4.36 (d, *J* = 12.8 Hz, 4H), 4.71 (t, *J* = 4.8 Hz, 4H), 4.85 (s, 4H), 4.91 (t, *J* = 4.8 Hz, 4H), 6.92-6.95 (m, 4H), 7.01 (s, 4H), 8.27 (brs, 2H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 31.3, 31.8, 33.9, 34.2, 59.2, 78.9, 79.2, 79.4, 79.6, 125.5, 125.8, 127.7, 133.2, 169.0; IR (KBr): 3393, 3309, 2961, 2869, 1653, 1636, 1486, 1363, 1193, 971 cm^{-1}; MS m/z: HRMS (ESI) Calcd: for $C_{56}H_{78}N_2NaO_{10}$ ([M+Na]$^+$): 961.5554, found: 961.5598.

25,27-bis[N,N-di(2-hydroxyethyl)aminocarbonylmethoxyl]-calix[4]arene-26,28-diol(**3e**): White powder, purified by column chromatography (SiO_2, 1:1 petroleum ether/ethyl acetate), Mp=225.7- 227.4 ℃, yield: 67 %; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 3.35 (d, J = 8.4 Hz, 4H), 3.44-3.48 (m, 8H), 3.57-3.59 (m, 8H), 4.40 (d, *J* = 8.4Hz, 4H), 4.74(t, *J*=3.6 Hz, 2H), 4.89 (s, 4H), 4.93 (t, *J* = 3.6 Hz, 2H), 6.54(t, *J* = 5.2 Hz, 2H), 6.74 (t, *J* = 5.2 Hz, 2H), 6.98 (d, *J* = 5.2 Hz, 4H), 7.08 (d, *J* = 4.8 Hz, 4H), 8.41 (s, 2H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 31.2, 59.0, 59.1, 128.1, 129.0, 129.2, 134.2, 154.2, 168.9; IR (KBr) υ: 3385, 1653, 1635, 1558, 1466, 1437, 1194, 1050, 755 cm^{-1}; MS m/z: HRMS (ESI) Calcd: for $C_{40}H_{46}N_2NaO_{10}$ ([M+Na]$^+$): 737.3045, found: 737.3087.

5,11,17,23,29,35-hexa-*tert*-butyl-37,38,39,40,41,42-hexa[N-(2-hydroxyethyl)aminocarbonylmethoxyl]-calix[6]arene(**3f**): White powder, purified by column chromatography (SiO_2, 6:1 dichloromethane/methanol), Mp > 250℃, yield: 68 %; ^{1}H NMR (DMSO-d_6, 400 MHz） δ: 1.00 (s, 54H), 3.16 (brs, 12H), 3.40 (brs, 6H), 4.00 (brs, 24H), 4.72 (t, *J* = 5.2 Hz, 6H), 6.88 (s, 12H), 7.92 (s, 6H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 29.9, 31.6, 34.3, 60.3, 72.3, 126.2, 132.8, 146.4, 152.8, 168.5; IR (KBr) υ: 3415, 2961, 2868, 1668, 1653, 1363, 1116, 874 cm^{-1}; MS m/z: HRMS (ESI) Calcd: for $C_{90}H_{126}N_6NaO_{18}$ ([M+Na]$^+$): 1601.9025, found: 1601.9038.

37,38,39,40,41,42-hexa[N-(2-hydroxyethyl)aminocarbonylmethoxyl]calix[6]arene(**3g**): White powder, purified by column chromatography (SiO_2, 10:1 ethyl acetate/methanol), Mp > 250℃, yield: 72 %; ^{1}H NMR（DMSO-d_6, 400 MHz）δ: 3.18 (s, 12H), 3.38 (s, 6H), 4.11 (d, *J* = 22.8 Hz, 24H), 4.73 (s, 6H), 6.81 (s, 12H), 6.93 (s, 6H), 7.95 (s, 6H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 29.8, 41.6, 56.5, 60.1, 72.2, 125.2, 129.4, 134.0, 154.7, 168.2; IR (KBr) υ: 3420, 3303, 2960, 1662, 1653, 1558, 1480, 1190, 1041, 974 cm^{-1}; MS m/z: HRMS (ESI) Calcd: for $C_{66}H_{78}N_6NaO_{18}$ ([M+Na]$^+$): 1265.5270, found: 1265.5247.

5,11,17,23,29,35-hexa-*tert*-butyl-37,38,39,40,41,42-hexa[N,N-di(2-hydroxyethyl)aminocarbonylmethoxyl]-calix[6]arene(**3h**). White powder, purified by column chromatography (SiO_2, 1:2 dichloromethane/methanol), Mp > 250℃, yield: 96.82%; ^{1}H NMR(DMSO-d_6, 400 MHz) δ: 0.94, 1.17 (2s, 54H), 3.30-3.53(m, 42H), 3.92 (d, J = 34.8 Hz, 12H), 4.57-4.87 (m, 16H), 6.74-7.10 (m, 12H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 30.0, 31.7, 34.2, 34.3, 34.4, 58.4, 59.4, 132.9, 146.0, 169.1; IR (KBr) υ: 3385, 2956, 2867, 1653, 1635, 1482, 1189,1049, 874 cm^{-1}; MS m/z: HRMS (ESI) Calcd: for $C_{78}H_{102}N_6NaO_{24}$ ([M+Na]$^+$): 1529.6843, found: 1529.6818.

37,38,39,40,41,42-hexa[N,N-di(2-hydroxyethyl)aminocarbonylmethoxyl]calix[6]arene (**3i**): White powder, purified by column chromatography (SiO_2, 1:2 dichloromethane/methanol), Mp > 250℃, yield: 58 %; ^{1}H NMR（DMSO-d_6, 400 MHz）δ: 3.36-3.43 (m, 24H), 3.44-3.50 (m, 24H), 4.05 (s, 12H), 4.72-4.76 (m, 12H), 4.94 (t, J = 5.2Hz, 6H), 6.76-6.92 (m, 18H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 30.0, 59.1, 59.3, 60.2, 61.0, 71.7, 124.6, 129.2, 134.4, 155.7, 168.6; IR (KBr) υ: 3377, 2955, 1657, 1645, 1481, 1194, 1045, 872 cm^{-1}; MS m/z: HRMS (ESI) Calcd: for $C_{78}H_{102}N_6Na_2O_{24}$ ([M+2Na]$^{2+}$): 1889.0497, found: 945.0259.

5,11,17,23,29,35,41,47-octa-*tert*-butyl-49,50,51,52,53,54,55,56-octa[N-(2-hydroxyethyl)aminocarbonylmethoxyl]calix[8] arene(**3j**). purified by column chromatography (SiO_2, 6:1 dichloromethane/methanol), White powder: Mp > 250℃, yield: 53 %; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 1.00 (s, 72H), 3.15 (brs, 16H), 3.38 (s, 16H), 4.00 (brs, 24H), 4.71 (brs, 8H), 6.87 (s, 16H), 7.91 (s, 8H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 31.5, 34.3, 41.6, 60.1, 72.0, 132.7, 146.3, 152.7, 168.4; IR (KBr) υ: 3413, 2960,

2865, 1667, 1653, 1558, 1539, 1476, 1363, 1189, 668 cm^{-1}; MS m/z: HRMS (ESI) Calcd: for $C_{120}H_{168}N_8NaO_{24}$ ($[M+Na]^+$): 2128.2069, found: 2128.2132.

49,50,51,52,53,54,55,56-octa[N-(2-hydroxyethyl)aminocarbonylmethoxyl]calix[8]arene(**3k**):White powder, purified by column chromatography (SiO_2, 7:1 ethyl acetate/methanol), Mp > 250℃, yield: 60%; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 3.20-3.40 (m,32H), 4.00-4.25 (m, 32H), 4.71 (s, 8H), 6.66-6.93 (m, 24H), 7.86-8.39 (m, 8H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 29.7, 29.9, 30.4, 41.6, 60.1, 60.1, 71.9, 72.2, 72.3, 124.9, 125.1, 127.4, 128.4, 128.8, 129.4, 129.5, 133.2, 134.3, 153.5, 154.4, 154.6, 154.9, 168.2, 168.2, 168.4; IR (KBr) υ: 3409, 2960, 1663, 1637, 1558, 1480, 1362, 1189, 874 cm^{-1}. MS m/z: HRMS (ESI) Calcd: for $C_{88}H_{104}N_8Na_2O_{24}$ ($[M+2Na]^{2+}$): 1702.6959, found: 851.3514.

5,11,17,23,29,35,41,47-octa-*tert*-butyl-49,50,51,52,53,54,55,56-octa[N,N-di(2-hydroxyethyl)aminocarbonylmethoxyl]calix[8]arene (**3l**): White powder, purified by column chromatography (SiO_2, 1:3 dichloromethane/methanol), Mp > 250℃, yield: 25 %; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 1.09, 1.17 (2s, 72H), 3.31-3.54 (m, 40H), 3.92 (d, J =3.9Hz, 16H), 4.79 (d, J =55.6Hz, 16H), 6.82-7.03 (m, 16H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 30.7, 31.7, 34.2, 58.0 59.6, 60.6, 71.8, 126.2, 133.3, 146.3, 150.9, 153.1, 169.4; IR (KBr) υ: 3411, 2961, 2869, 1648, 1633, 1482, 1363, 1202, 874 cm^{-1}; MS m/z: HRMS (ESI) Calcd: for $C_{136}H_{200}N_8Na_2O_{32}$ ($[M+2Na]^{2+}$): 2503.4064, found: 1251.7051.

49,50,51,52,53,54,55,56-octa[N,N-di(2-hydroxyethyl)aminocarbonylmethoxyl]-calix[8]arene(**3m**).White powder, purified by column chromatography (SiO_2, 1:1 dichloromethane/methanol), Mp > 250℃, yield: 37%; ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 3.43-3.50 (m, 64H), 3.93-4.03 (m, 16H), 4.75 (brs, 16H), 6.64-7.07 (m, 24H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 30.2, 57.9, 59.4, 71.7, 73.4, 124.1, 124.7, 129.3, 134.1, 134.4, 134.7, 155.9, 156.4, 169.0, 173.9; IR (KBr) υ: 3331, 2949, 1632, 1362, 1192, 1045, 868 cm^{-1}. MS m/z: HRMS (ESI) Calcd: for $C_{104}H_{136}N_8Na_2O_{32}$ ($[M+2Na]^{2+}$): 2054.9056, found: 1027.4544.

4.2.Crystallography

The single crystal of **3a** was obtained in ethanol and the single crystal structure was determined on Bruker Smart Apex X-single crystal diffractometer. The data were processed with HKL2000. The structure was solved by direct methods of SHELX86 and subsequent Fourier-difference synthesis and refined by full-matrix least-squares on F^2 with SHELXS-9737[45]. No absorption correction was done. All non-hydrogen atoms were refined with anisotropic displacement parameters.

4.3.Cellular Assay

Cell culture: A549 cells (Human lung cancer cells), SKOV3 cells (Human ovarian cancer), SW1990 cells (Human pancreatic cancer), Hela cells (Human cervical cancer), Raji cells (Human Burkitt's shower Pakistan tumor), MDA-MB-231 cells (Human breast cancer) were kindly provided by WeiFang Caleb Pharmaceuticals, Inc. A549 cells were cultured in culture Ham's F12K medium containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine and 1.5g/L sodium bicarbonate. SKOV3 cells were cultured on Cell culture flask using 10 % fetal bovine serum, 1.5 mM L-glutamine and 2.2 g/L sodium bicarbonate in McCoy's 5a medium. SW1990 cells and MDA-MB-231 cells were cultured in Leibovitz's L-15 medium with 10% (v/v) fetal bovine serum and 2 mM L-glutamine. Hela cells were cultured on Cell culture flask using 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES and 1.0 mM sodium pyruvate in RPMI 1640 medium supplemented with 0.5 mg/ml G418and 10% fetal bovine serum. Raji cells were cultured in RPMI 1640 medium with 1.5 g/L sodium bicarbonate, 2.5 g/L glucose and 0.11 g/L sodium pyruvate, supplemented with 10 % fetal bovine serum.

4.4. Cytotoxicity assay

In this experiment, all the adherent cells used SRB method. All suspension cells used MTT method.

SRB assay: All cells were cultured in culture medium containing 10 % fetal calf serum, and been in the logarithmic growth phase. All cell types were seeded in 96-well culture plate at a concentration of 1-5×10^4 cells per well at 37℃ in 5% CO_2 incubator for 24 h. The cells were then exposed to 10 drug concentrations of calixarene derivatives for 72 h with each concentration located three wells. Then the cells were fixed with trichloroacetic acid (TCA). After washing, SRB working solution was added to the cells to clean them. SRB combined with protein was dissolved in Tris base. OD values were measured for each well with SPECTRA max 190 Cell microplate reader under 565 nm wavelength.

MTT assay: All cells were cultured in culture medium containing 10% fetal calf serum, and been in the logarithmic growth

phase. All cell types were seeded in 96-well culture platet at a concentration of 1-5 $\times 10^4$ cells per well at 37℃ in 5% CO_2 incubator for 24 h. The cells were then exposed to 10 drug concentrations of calixarene derivatives for 72 h with each concentration located three wells. Then MTT solution was added to each well, the cells were placed in incubator for 4 h. Each well was removed 100 ul solution, added 100ul of three Joint fluid (HCl + isopropanol + SDS), and placed in the incubator overnight. Next day, the cells were labeled in SPECTRA max 190Cell microplate reader, and OD values were measured under 550 nm wavelength.

4.5.Flow cytometry for cell cycle analysis

fter incubation for 48 h at 37℃ in an atmosphere of 5 % CO_2, SKOV3 cells were detached by trypsinization, collected, washed three times with PBS and fixed in ice-cold ethanol for overnight. The cells were washed twice with PBS and resuspended in working solution (10 μg/mL propidiumiodide (PI), 10 μg /mL RNase) and incubated at 37℃ in the dark for 30 min. Cell cycle analysis was performed by analysis of PI staining using a FACSC alibur flow cytometer.

4.6. Immunofluorescence assay

SKOV3 cells were grown in 24-well plates, treated with **3a**. After 24 h, cells were fixed with 4 % paraformaldehyde for 20 min and perforated with 0.3 % Triton X-100 for 10 min. The cells were blocked with sheep serum followed by incubation with P53/Bax/Bcl-2/Caspase-3 antibody at 4℃overnight. The cells were incubated with the relevant secondary antibody conjugated with goat antimouse or antirabbit antibody for 1h, and counterstained with Hoechst 33342 for 15 min. Finally, Cells were mounted on glass slide and images were examined and captured under a fluorescence microscope (Olympus, BX43F).

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Conflict of Interest

The authors declare no conflict of interest.

Author contributions statement

L. An, L. L. Han and X. N. Peng synthesized all the compounds; J. Sun determined and analyzed the single crystal. Y.S.Xue optimized the Molecular structure. L. L. Han performed the flow cytometry for cell-cycle analysis and Immunofluorescence staining. All authors extensively discussed the results; the paper was written by L. An, C. G. Yan, with the help of Y. G. Zheng, X. K. Gu. All authors reviewed the manuscript.

Additional information

The detailed spectroscopic data including crystallographic data (CIF) of compounds are available. Single crystal data for compounds **3a** has been deposited in the Cambridge Crystallographic Data Center and assigned to the following deposition number CCDC 1450999.

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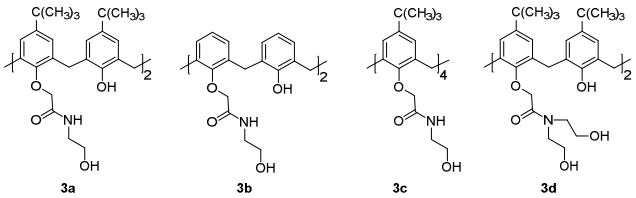


Figure1. Structural representations of calix[4]arene polyhydroxyamine derivatives **3a–3d**

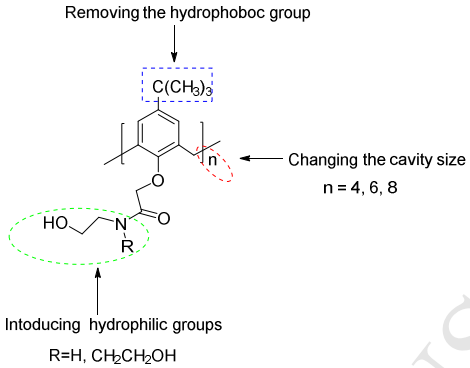
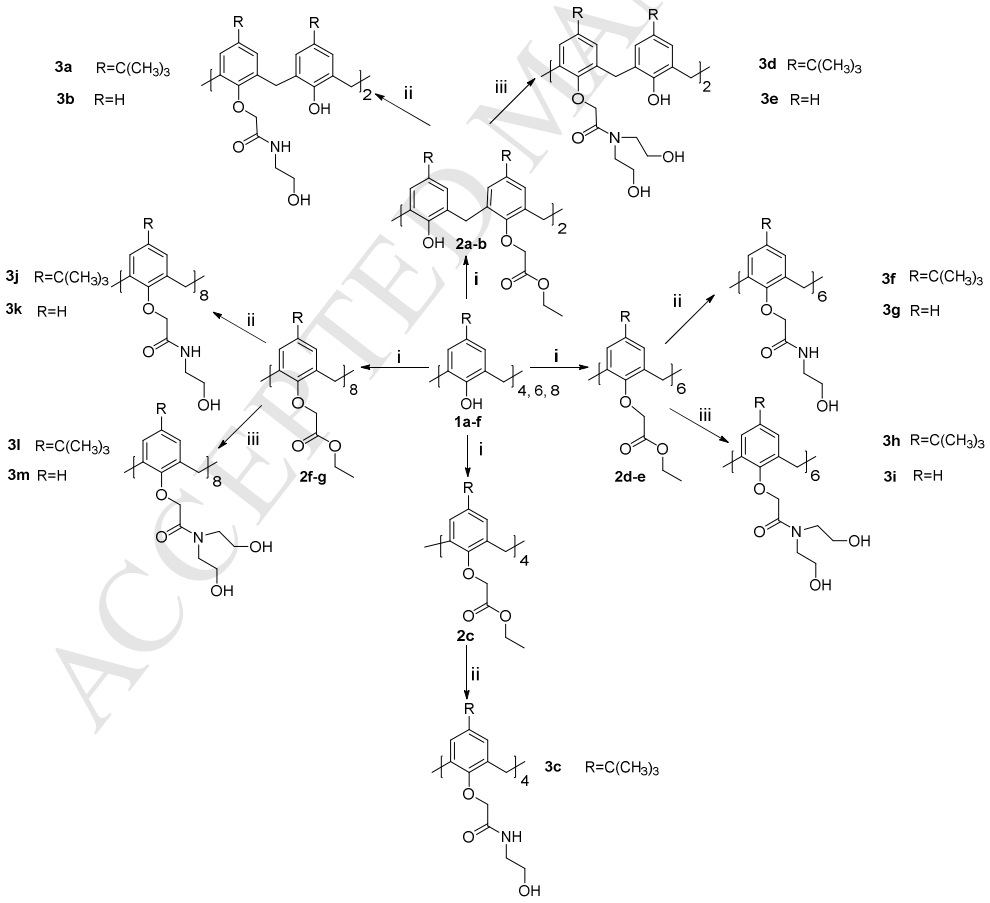


Figure 2. Structural optimization design of calix[n]arene



Scheme1. Synthesis of calix[n]arene polyhydroxyamine derivatives **3a–3m**

Reagents and conditions: (i) $ClCH_2COOC_2H_5$, K_2CO_3, acetone, ultrasonic irradiation; (ii) $NH_2CH_2CH_2OH$, ethanol/toluene,reflux; (iii) $NH(CH_2CH_2OH)_2$,

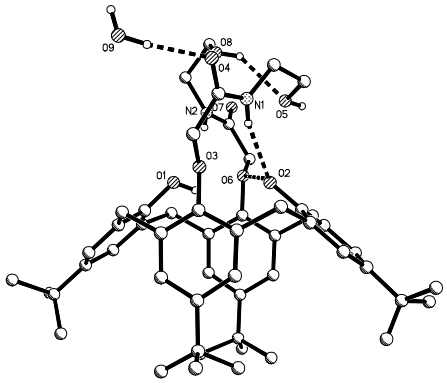


Figure 3. Single crystal structure of **3a** (hydrogen atoms on the skeletons were omitted for clarity)

Table 1. Crystal data for Compound **3a**

Phase	**3a**
Empirical formula	$C_{52}H_{72}N_2O_9$
Formula weight	869.12
Temperature	296(2)
Wavelength/nm	0.71073
Crystal system	Triclinic
space group	P -1
a (Å)	9.937(4)
b (Å)	12.228(4)
c (Å)	22.273(6)
α(°)	87.276(10)
β(°)	82.963(13)
γ(°)	72.794(13)
Volume(Å3)	2565.7(14)
Z	2
F(000)	940
Crystal size (mm)	0.36 x 0.34 x 0.28
Calculated density (g.cm-3)	1.125
Absorption coefficient (mm-1)	0.076
θ range (°)	0.92 to 25.00
Limiting indices	-11 < =h < =11, -14 < =k < =14, -26 < =l < =26
Reflections collected / unique	29477 / 8702 [R(int.) = 0.1267]
Completeness to theta	96.3 %
Max. and min. transmission	0.979 and 0.973
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	8702 / 72 / 601
Goodness-of-fit on F^2	1.038
Final R indices [I > 2sigma(I)]	R_1 = 0.1203, wR_2 = 0.2627
R indices (all data)	R_1 = 0.2326, wR_2 = 0.3168
Largest diff. peak and hole(e.Å-3)	0.589 and -0.456

Table 2. Single concentration inhibition of calixarenes **3a–3m** on cancer cell viability

Compound	Single concentration inhibition (%)[a]					
	A549	**SKOV3**	**SW1990**	**Hela**	**Raji**	**MDA-MB-231**
3a	89	97	95	100	100	99
3b	13	86	10	46	29	57
3c	93	95	99	100	100	99
3d	16	42	59	95	77	95
3e	7	34	4	38	34	22
3f	7	24	4	40	38	12
3g	7	10	3	6	18	4
3h	0	26	26	0	14	0
3i	5	19	0	9	6	22
3j	8	26	5	23	24	12
3k	2	1	4	32	7	27
3l	0	25	5	10	11	11
3m	6	25	2	18	11	13

[a] the concentration of the calixarenes **3a–3m** was 10 μM

Table3. IC_{50} values (μM) of calixarenes **3a–3d** on various cancer cells

Cancer cells	IC_{50} (μM)			
	3a	**3b**	**3c**	**3d**
A549	4.0	--	3.7	--
SKOV3	2.8	2.3	5.1	--
SW1990	4.3	--	3.3	11.3
Hela	2.7	--	7.1	8.1
Raji	5.0	--	4.7	11.2
MDA-MB231	5.3	1.6	3.4	5.2

"--"means no detected

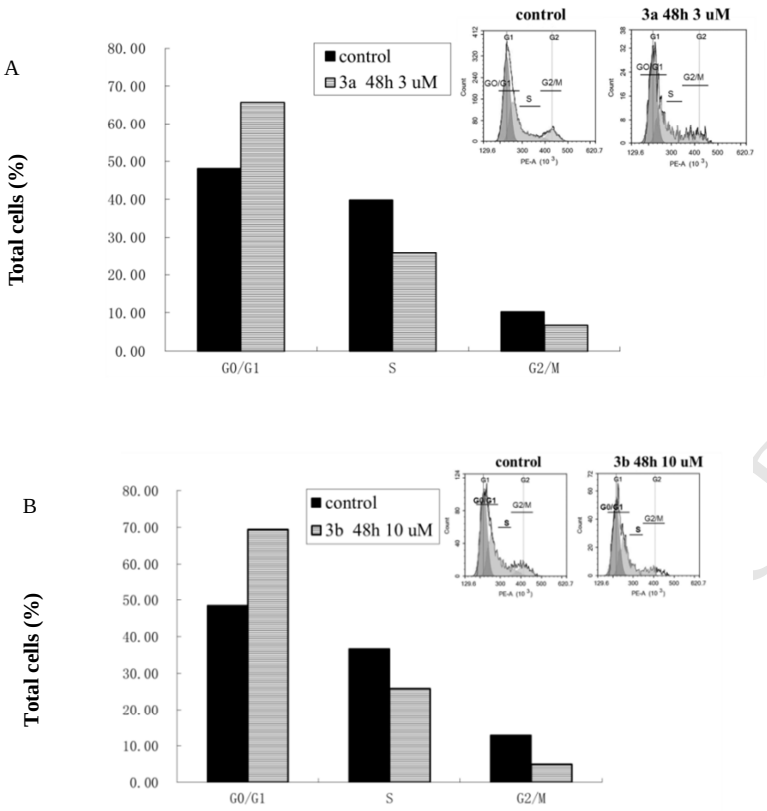


Figure 5. The amount of SKOV3 cells each cell cycle phase (G0/G1 phase, S phase and G2/M) after treatment with specified concentrations of **3a** (A) and **3b** (B) for 48 h was determined by propidium iodide staining followed by flow cytometry.

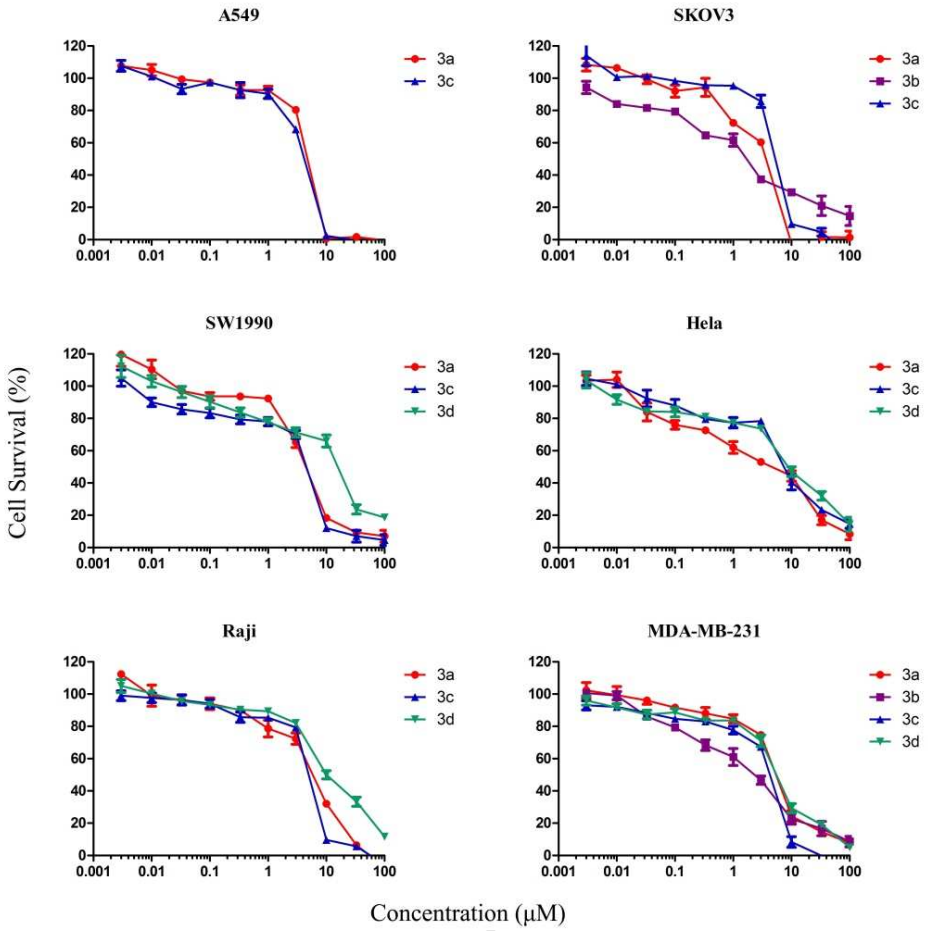
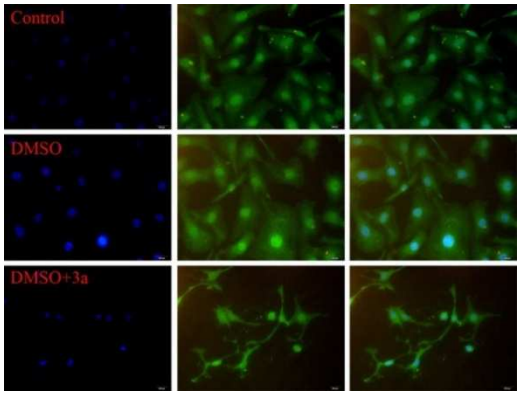
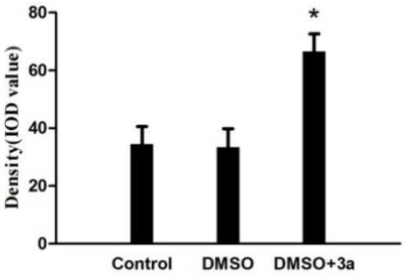
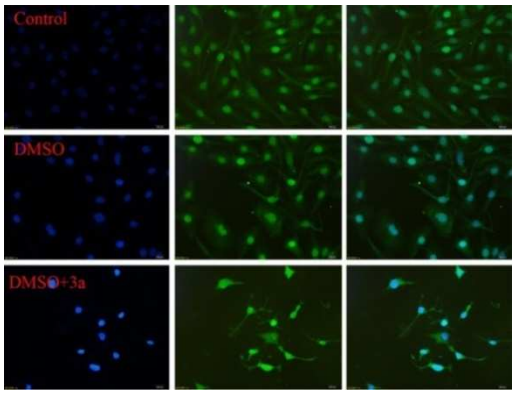
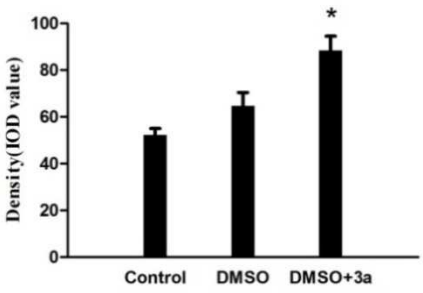


Figure 4. Cytotoxicity effects of calix[4]arenes **3a–3d**. Cell viability of A549, SKOV3, SW1990, Hela, Raji as well as MDA-MB231cells was measured in the presence of various concentrations of calixarenes.









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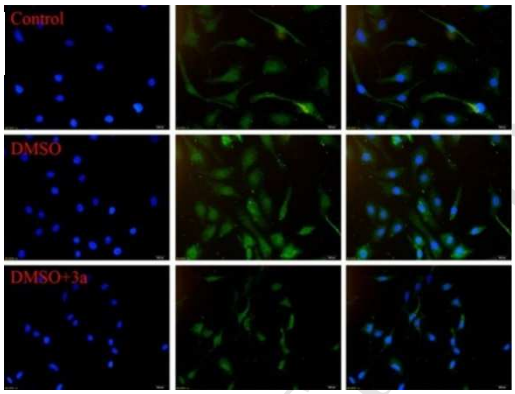

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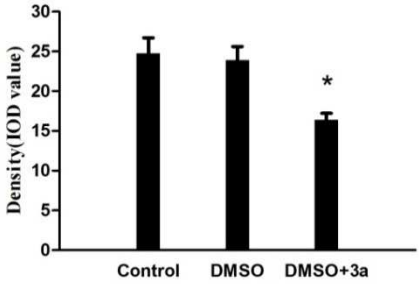

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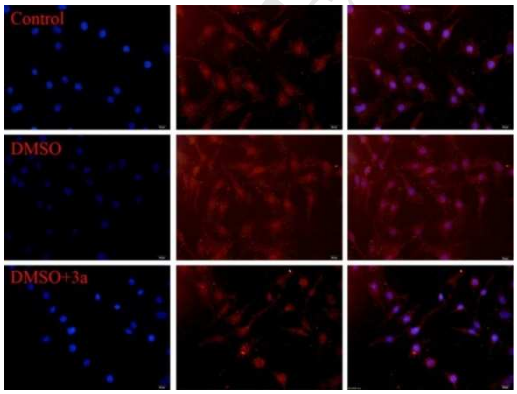

Fig.**6** Effects of apoptotic related proteins expression on the apoptosis of treated and untreated SKOV3 cells. Caspase-3 (A), P53 (C), Bcl-2 (E), Bax (F) protein antibody and fluorescein-conjugated second antibody were used to visualize corresponding protein, and Hoechst 33342 was used to stain cell nuclei (blue).The levels of Caspase-3 (B), P53 (D) are increased in SKOV3 cells, the level of Bcl-2 (E) is decreased with Bax protein no changes**.**

Highlights

- Novel calix[n]arene (n = 4, 6, 8) polyhydroxyamine derivatives (**3a–3m**) were designed and afforded, including a single crystal of **3a**.
- Cell viability assay were evaluated in the six cell lines (A549, SKOV3, SW1990, Hela, Raji and MDA-MB-231).
- Calix[4]arenes **3a–3d** indicated good anti-tumor activity with IC_{50} values ranging from 1.6 μM to 11.3 μM.
- Cell cycle analysis and Immunofluorescent staining indicated that **3a** can be further studied as a potential anticancer agent.