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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and anticancer activity evaluation of novel oxacalix[2]arene[2]pyrimidine derivatives

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Abstract

A series of novel oxacalix[2]arene[2]pyrimidine derivatives were synthesized, and their antitumor activities against HeLa, MCF7, HepG2, and A549 human cancer cell lines were evaluated using an MTT assay. Some of the synthesized compounds exhibited considerable anti-proliferative activity against the human cancer cell lines. Compound **5l**, which contains an ethanolamine moiety, exhibited the strongest inhibitory activity against HepG2 with an IC_{50} value of 12.37 μM. Moreover, a cell apoptosis assay indicated that the anti-proliferative activity of **5l** might be attributed to its induction of apoptosis. Our report highlights the potential anticancer efficacy of novel oxacalix[2]arene[2]pyrimidines.

Keywords Oxacalix[2]arene[2]pyrimidine · Ethanolamine · Anticancer · Apoptosis

Introduction

Cancer is the second leading cause of death worldwide, and as the global population ages and changes its lifestyle, cancer mortality will inevitably increase (Bray et al. 2018; Vineis and Wild 2014). According to a recent report by the World Health Organization (WHO), an estimated 9.6 million people died of cancer in 2018, and this number will increase to 15 million by 2030. Although recent studies have identified a significant number of novel anticancer drugs and proven their efficacy in treatment for specific cancers, cancer mortality remains high due to cancer drug resistance (Olgen 2018; Housman et al. 2014). Thus, it is still urgent to develop novel, effective, and safe chemotherapeutic agents for cancer treatment.

Calixarene is an important type of macrocyclic molecule with unique three-dimensional conformational and cavity structures (Steed 2015). In the past few decades, calixarenes have been widely exploited as molecular platforms for molecular recognition, self-assembly, catalysis, nanotechnology, and drug discovery due to their flexible macrocyclic backbones and straightforward functionalization at both the upper and lower rims (Mutihac et al. 2011; Zadmard and Schrader 2006; Fa et al. 2014; Caio et al. 2014; Li et al. 2014). In particular, their potential applications as antimicrobial agents, antiviral agents, anti-tubercular agents, and anticancer agents in biomedical fields have attracted significant attention (Soares et al. 2014; Dings et al. 2013, 2014; Pelizzaro-Rocha et al. 2013; An et al. 2016). However, the type of molecules reported to exhibit anticancer activity are mainly conventional calixarenes in which the aromatic rings are linked by methylene units ($\mathbf{a_1}$–$\mathbf{a_4}$, Fig. 1a) (Dings et al. 2013, 2016; Pelizzaro-Rocha et al. 2013; An et al. 2016). Only a few heterocalixaromatics/heteroatom-bridged calix(het)-arenes, such as azacalix[2]arene[2]pyrimidines ($\mathbf{a_5}$), sulphonyl[4]calixarenes ($\mathbf{a_6}$), and calix[4]pyrroles ($\mathbf{a_7}$), have been reported to be effective in cancer treatment, and research on the biological activity of oxygen-bridged calix(het)-arenes is rare (Fig. 1a) (Addepalli et al. 2018; Buldenkoa et al. 2017; Geretto et al. 2018).

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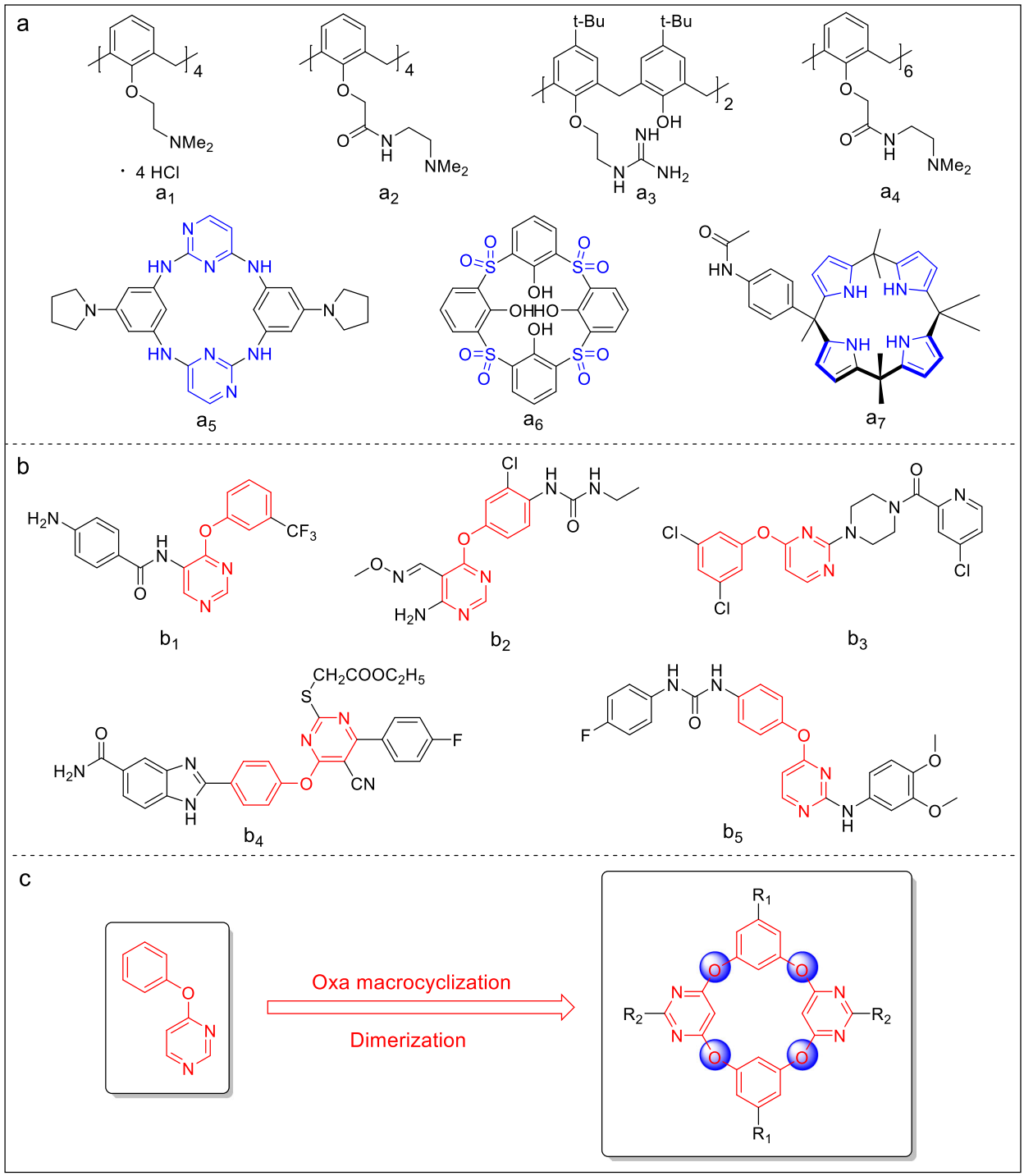


Fig. 1 **a** Structures of anticancer calixarenes reported in the literature. **b** Structures of anticancer pyrimidinyloxyphenyl derivatives reported in the literature. **c** Our design strategy for novel oxacalix[2]arene[2]pyrimidines

Pyrimidine is a significant six-membered heterocyclic compound that contains two nitrogen atoms at the first and third positions and serves as the building blocks for DNA and RNA (Kaur et al. 2015). Studies on pyrimidine and its derivatives suggest that they have a wide range of pharmacological effects, exhibiting anticancer, antimalarial, anti-HIV, antiviral, antimicrobial, and anti-inflammatory activities (Kumar et al. 2018; Kamchonwongpaisan et al. 2004; Kraljevic et al. 2012; Moty et al. 2016; Preetam and Nath 2015). Among the various derivatives,

pyrimidinyloxyphenyl derivatives have attracted considerable attention due to their effectiveness in cancer therapy and favorable pharmaceutical properties. Over the last decade, a large number of compounds containing pyrimidinyloxyphenyl fragments, such as **b_1–b_5** (Fig. 1b), have been identified as potential anticancer agents (Elkamhawy et al. 2017; Huang et al. 2011; Yao et al. 2017; Galal et al. 2018; Gao et al. 2016). Based on this research, introducing pyrimidinyloxyphenyl to a calixarene skeleton might be an efficient strategy for the development of novel antitumor agents.

Due to the potential anticancer activity of pyrimidinyloxyphenyls and our interest in oxygen-bridged calix(het)-arenes, we designed and synthesized a series of novel oxacalix[2]arene[2]pyrimidines, and evaluated their potential as antitumor agents (Fig. 1c). The antitumor activities of these compounds were assessed with four different human tumor cell lines, namely, the human cervical cancer cell line HeLa, the human breast cancer cell line MCF7, the human hepatocarcinoma cell line HepG2, and the human non-small cell lung cancer cell line A549. Furthermore, a cell apoptosis assay was performed to investigate the preliminary anticancer mechanism of the representative compound **5l**. To the best of our knowledge, this is the first report focusing on the biological activity of oxacalix[2]arene[2]pyrimidine derivatives.

Experimental procedures

Chemistry

All chemicals, reagents, and solvents used in the reaction were purchased from commercial sources and used without any further purification. The melting points were measured with an YRT-3 melting point apparatus in opened capillaries and were uncorrected. The nuclear magnetic resonance (NMR) spectra were obtained with a JNM-ECZR 400 MHz spectrometer in $CDCl_3$ or DMSO-d_6 at ambient temperature (400 MHz for ^1H and 100 MHz for ^{13}C). Tetramethylsilane (TMS) is used as the internal standard, chemical shifts (δ) are presented in parts per million (ppm) and coupling constants (J) are given in hertz (Hz). Moreover, the peak multiplicity is given as following abbreviations: s (singlet), d (doublet), dd (double doublet), t (triplet), q (quartet), and m (multiplet). IR spectra were carried out using a Nicolet FT-IR 8400 spectrometer. HRESIMS spectra were obtained with a (UHR-TOF) maXis 4G instrument. The reaction progress was monitored by thin-layer chromatography (TLC) on Merck silica gel (60-F254) plates. Column chromatography separations were carried out on silica gel (300–400 mesh, Qingdao Marine Chemical Ltd., Qingdao, China).

Synthesis of *4,6,16,18-Tetraaza-11,23-bis(methylformate)-5,17-bis(methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene* (3)

We successively added 4,6-dichloro-2-methylthiopyrimidine (**1**, 5.0 g, 25.6 mmol), methyl 3,5-dihydroxybenzoate (**2**, 4.30 g, 25.6 mmol), and 18-crown-6 (660 mg, 2.5 mmol) to a stirred solution of potassium carbonate (9.05 g, 65.5 mmol) in DMF (250 ml) at room temperature. The reaction mixture was then stirred under argon at 75 °C for 72 h. After cooling to room temperature, the reaction mixture was diluted with water (800 ml) and the resulting precipitate was collected by filtration, producing a white solid. Then, the solid was stirred in dichloromethane (400 ml), the insoluble material was filtered off, and the filtrate was evaporated under reduced pressure and purified by silica gel column chromatography (petroleum ether–ethyl acetate, 2/1, v/v) to give compound **3** as a white solid; yield: 34.5%; m.p. 223.5–224.5 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ: 7.59 (d, $J = 2.2$ Hz, 4H), 7.54 (t, $J = 2.2$ Hz, 2H), 5.98 (s, 2H), 3.81 (s, 6H), 2.50 (s, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 175.8, 171.8, 164.6, 152.9, 135.4, 121.4, 119.2, 82.8, 53.0, 14.4; HRESIMS *m/z* calcd. for $C_{26}H_{20}N_4O_8S_2$ $[M + H]^+$ 581.0795, found: 581.0798.

Synthesis of *4,6,16,18-Tetraaza-11,23-bis(formicacid)-5,17-bis(methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene* (4)

We added a solution of sodium hydroxide (0.488 g, 12.0 mmol) in water (20 ml) to a stirred solution of compound **3** (2.9 g, 2.5 mmol) in THF (40 ml). The reaction mixture was heated to reflux under ultrasonic irradiation for 4 h. After cooling to room temperature, THF was removed via evaporation under reduced pressure and the residue was acidified to a pH of 6–7 with 1 M HCl. The resulting precipitate was filtered, washed with water, and dried to give compound **4** as a white solid; yield: 94.2%; m.p. 226.5–227.5 °C; ^1H NMR (DMSO-d_6, 400 MHz) δ: 13.37 (s, 2H), 7.50 (s, 6H), 5.94 (t, $J = 5.5$ Hz, 2H), 2.50 (s, 6H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 173.2, 171.8, 166.0, 153.9, 135.8, 119.8, 117.9, 86.7, 14.1; HRESIMS *m/z* calcd. for $C_{24}H_{16}N_4O_8S_2$ $[M + H]^+$ 553.0482, found: 553.0495.

General procedure for synthesis of oxacalix[2]arene[2]pyrimidines (5a–5o)

To synthesize the target compounds **5a**–**5o**, we added HATU (0.237 g, 0.625 mmol), triethylamine (0.063 g, 0.625 mmol), and appropriate amine to a stirred solution of compound **4** (0.138 g, 0.25 mmol) in DMF (5 ml). Next, the mixture was stirred at room temperature until the reaction was complete as determined by TLC. The reaction mixture was then slowly added to 20 ml of ice water. The resulting precipitate was filtered, washed with water, dried, and purified by silica gel column chromatography

(dichloromethane–methyl alcohol, 10/1, v/v) to give compounds **5a**–**5o**. Yields, melting points, and spectroscopic data for oxacalix[2]arene[2]pyrimidines are listed as follows.

4,6,16,18-Tetraaza-11,23-bis(N-formylbutylamino)-5,17-bis (methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (5a)

White solid; yield: 18.13%; m.p. 176.5–177.5 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) *δ:* 8.52 (t, $J = 5.2$ Hz, 2H), 7.54 (d, $J = 2.0$ Hz, 4H), 7.41 (t, $J = 2.0$ Hz, 2H), 5.89 (s, 2H), 3.18 (dd, $J = 12.8$, 7.2 Hz, 4H), 2.50 (s, 6H), 1.45–1.38 (m, 4H), 1.27–1.19 (m, 4H), 0.83 (t, $J = 7.2$ Hz, 6H); ^{13}C NMR (DMSO-d_6, 100 MHz) *δ:* 173.2, 171.9, 164.1, 153.6, 139.2, 118.0, 116.1, 86.4, 29.2, 22.3, 14.4, 14.1; IR (KBr) *v*: 3350, 2930, 1647, 1572, 1549, 1439, 1364, 1321, 1290, 1165, 1142, 1024, 806; HRESIMS *m/z* calcd. for $C_{32}H_{34}N_6O_6S_2$ $[M + H]^+$ 663.2054, found: 663.2069.

4,6,16,18-Tetraaza-11,23-bis(N-formylamylamino)-5,17-bis (methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (5b)

White solid; yield: 17.4%; m.p. 143.5–144.5 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) *δ:* 8.57 (t, $J = 5.6$ Hz, 2H), 7.58 (d, $J = 2.0$ Hz, 4H), 7.45 (t, $J = 2.0$ Hz, 2H), 5.95 (s, 2H), 3.22 (dd, $J = 12.8$, 6.4 Hz, 4H), 2.55 (s, 6H), 1.47–1.44 (m, 4H), 1.27–1.24 (m, 8H), 0.85 (t, $J = 6.8$ Hz, 6H); ^{13}C NMR (DMSO-d_6, 100 MHz) *δ:* 173.2, 171.9, 164.1, 153.6, 139.2, 118.0, 116.1, 86.4, 29.2, 29.1, 22.4, 14.4, 14.1; IR (KBr) *v*: 3325, 2930, 1649, 1572, 1549, 1439, 1362, 1288, 1163, 1140, 1024, 804, 675; HRESIMS *m/z* calcd. for $C_{34}H_{38}N_6O_6S_2$ $[M + H]^+$ 691.2367, found: 691.2331.

4,6,16,18-Tetraaza-11,23-bis(N-formylhexylamino)-5,17-bis (methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (5c)

White solid; yield: 20.0%; m.p. 185.5–186.5 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) *δ:* 7.34 (s, 4H), 6.89 (s, 4H), 5.04 (s, 2H), 3.31 (s, 4H), 2.61 (s, 6H), 1.50 (s, 4H), 1.27 (s, 12H), 0.87 (s, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) *δ:* 175.6, 171.4, 165.2, 152.7, 140.3, 118.7, 116.7, 82.7, 40.4, 31.4, 29.3, 26.6, 22.5, 14.3, 14.0; IR (KBr) *v*: 3363, 2928, 1660, 1570, 1405, 1362, 1290, 1164, 1139, 1022, 896, 803; HRESIMS *m/z* calcd. for $C_{36}H_{42}N_6O_6S_2$ $[M + Na]^+$ 741.2499, found: 741.2500.

4,6,16,18-Tetraaza-11,23-bis(N-formyloctylamino)-5,17-bis (methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (5d)

White solid; yield: 21.5%; m.p. 125.5–126.5 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) *δ:* 7.33 (s, 4H), 6.90 (s, 4H), 5.04 (s, 2H), 3.30 (s, 4H), 2.61 (s, 6H), 1.50 (s, 4H), 1.25 (s, 20H), 0.87 (s, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) *δ:* 175.6, 171.3, 165.2, 152.7, 140.3, 118.7, 116.7, 82.6, 40.4, 31.7, 29.3, 29.1, 26.9, 22.6, 14.2, 14.1; IR (KBr) *v*: 3336, 2927, 1646, 1570, 1408, 1362, 1290, 1164, 1139, 1024, 893, 804; HRESIMS *m/z* calcd. for $C_{40}H_{50}N_6O_6S_2$ $[M + Na]^+$ 797.3125, found: 797.3135.

4,6,16,18-Tetraaza-11,23-bis(N-formylcyclopentylamino)-5,17-bis(methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (5e)

Pale yellow solid; yield: 15%; m.p. 132.5–133.5 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) *δ:* 8.38 (d, $J = 7.2$ Hz, 2H), 7.62 (d, $J = 2.0$ Hz, 4H), 7.44 (t, $J = 2.0$ Hz, 2H), 5.86 (s, 2H), 4.18 (dd, $J = 13.2$, 6.8 Hz, 2H), 2.55 (s, 6H), 1.85–1.80 (m, 4H), 1.66 (s, 4H), 1.53–1.46 (m, 8H); ^{13}C NMR (DMSO-d_6, 100 MHz) *δ:* 173.2, 171.9, 163.8, 153.5, 139.2, 118.2, 116.2, 86.2, 51.6, 32.5, 24.1, 14.1; IR (KBr) *v*: 3304, 2961, 1649, 1570, 1551, 1439, 1362, 1290, 1163, 1138, 1022, 806; HRESIMS *m/z* calcd. for $C_{34}H_{34}N_6O_6S_2$ $[M + Na]^+$ 709.1873, found: 709.1876.

4,6,16,18-Tetraaza-11,23-bis(N-formylbenzylamino)-5,17-bis (methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (5f)

White solid; yield: 17.4%; m.p. 229.5–230.5 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) *δ:* 9.22 (t, $J = 6.0$ Hz, 2H), 7.69 (d, $J = 1.2$ Hz, 4H), 7.52 (s, 2H), 7.39–7.30 (m, 10H), 6.05 (s, 2H), 4.50 (d, $J = 5.2$ Hz, 4H), 2.60 (s, 6H); ^{13}C NMR (DMSO-d_6, 100 MHz) *δ:* 173.3, 171.9, 164.5, 153.8, 139.7, 139.0, 128.9, 128.0, 127.5, 118.1, 116.4, 86.6, 43.4, 14.2; IR (KBr) *v*: 3371, 2928, 1663, 1570, 1551, 1362, 1290, 1165, 1140, 1022, 762; HRESIMS *m/z* calcd. for $C_{38}H_{30}N_6O_6S_2$ $[M + H]^+$ 731.1741, found: 731.1749.

4,6,16,18-Tetraaza-11,23-bis(N-formylphenylamino)-5,17-bis (methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (5g)

Pale yellow solid; yield: 15.8%; m.p. 226.5–227.5 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) *δ:* 10.28 (s, 2H), 7.72 (s, 4H), 7.68 (d, $J = 8.0$ Hz, 4H), 7.50 (s, 2H), 7.30 (t, $J = 8.0$ Hz, 4H), 7.07 (t, $J = 7.2$ Hz, 2H), 5.96 (s, 2H), 2.53 (s, 6H); ^{13}C NMR (DMSO-d_6, 100 MHz) *δ:* 173.3, 171.9, 163.6, 153.7, 139.4, 139.2, 129.2, 124.6, 121.1, 118.6, 116.7, 86.6, 14.2; IR (KBr) *v*: 3367, 2928, 1664, 1572, 1549, 1441, 1364, 1283, 1161, 1142, 1026, 760; HRESIMS *m/z* calcd. for $C_{36}H_{26}N_6O_6S_2$ $[M + H]^+$ 703.1428, found: 703.1423.

4,6,16,18-Tetraaza-11,23-bis(N-formyl-4-fluorophenylamino)-5,17-bis(methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (5h)

Pale yellow solid; yield: 21.7%; mp 228.5–229.5 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) *δ:* 10.39 (s, 2H), 7.74 (dd, $J = 10.8$, 5.2 Hz, 8H), 7.55 (t, $J = 2.4$ Hz, 2H), 7.19

(t, $J = 8.8$ Hz, 4H), 6.02 (s, 2H), 2.57 (s, 6H); ^{13}C NMR (DMSO-d_6, 100 MHz) *δ:* 173.3, 171.9, 163.5, 160.2, 157.8, 153.7, 139.2, 135.5, 123.0, 118.6, 115.7, 86.6, 14.2; IR (KBr) *v*: 3343, 2928, 1655, 1570, 1551, 1508, 1406, 1362, 1290, 1161, 1140, 1024, 806; HRESIMS *m/z* calcd. for $C_{36}H_{24}F_2N_6O_6S_2$ $[M + H]^+$ 739.1240, found: 739.1250.

4,6,16,18-Tetraaza-11,23-bis(N-formyl-4-methoxyphenylamino)-5,17-bis(methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (5i)

Pale yellow solid; yield: 21%; mp 230.5–231.5 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) *δ:* 10.20 (s, 2H), 7.74 (d, $J = 2.0$ Hz, 4H), 7.61 (d, $J = 9.2$ Hz, 4H), 7.52 (s, 2H), 6.91 (d, $J = 8.8$ Hz, 4H), 5.98 (s, 2H), 3.73 (s, 6H), 2.56 (s, 6H); ^{13}C NMR (DMSO-d_6, 100 MHz) *δ:* 173.3, 171.9, 163.0, 156.3, 153.6, 139.5, 132.1, 122.6, 118.5, 116.6, 114.3, 86.4, 55.7, 14.1; IR (KBr) *v*: 3416, 2951, 1661, 1572, 1551, 1512, 1364, 1283, 1163, 1024, 829; HRESIMS *m/z* calcd. for $C_{38}H_{30}N_6O_8S_2$ $[M + Na]^+$ 785.1459, found: 785.1470.

4,6,16,18-Tetraaza-11,23-bis(N-formyl-3,4-dimethoxyphenylamino)-5,17-bis(methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (5j)

Pale yellow solid; yield: 43.8%; mp 190.5–191.5 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) *δ:* 8.96 (s, 2H), 7.35 (s, 4H), 7.22 (s, 2H), 6.88 (s, 4H), 6.73 (d, $J = 9.2$ Hz, 2H), 5.02 (s, 2H), 3.84 (s, 6H), 3.76 (s, 6H), 2.61 (s, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) *δ:* 175.6, 171.0, 163.8, 152.5, 148.9, 146.4, 140.2, 130.6, 118.9, 117.1, 112.5, 111.1, 104.9, 82.0, 55.8, 14.1; IR (KBr) *v*: 3348, 2930, 1665, 1569, 1549, 1514, 1441, 1362, 1285, 1231, 1138, 1024, 802; HRESIMS *m/z* calcd. for $C_{40}H_{34}N_6O_{10}S_2$ $[M + H]^+$ 823.1851, found: 823.1871.

4,6,16,18-Tetraaza-11,23-bis(N-formyl-6-aminoquinolino)-5,17-bis(methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (5k)

Pale yellow solid; yield: 27.4%; m.p. 138.5–139.5 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) *δ*: 10.62 (s, 2H), 8.80 (dd, $J =$ 4.0, 1.2 Hz, 2H), 8.49 (s, 2H), 8.29 (d, $J = 7.2$ Hz, 2H), 7.99 (s, 4H), 7.82 (d, $J = 2.4$ Hz, 4H), 7.59 (d, $J = 2.0$ Hz, 2H), 7.48 (dd, $J = 8.4$, 4.4 Hz, 2H), 6.06 (s, 2H), 2.58 (s, 6H); ^{13}C NMR (DMSO-d_6, 100 MHz) *δ:* 173.4, 171.9, 163.8, 153.7, 149.9, 145.5, 139.1, 137.1, 136.2, 129.9, 128.6, 124.7, 122.3, 118.8, 117.2, 117.0, 86.5, 14.2; IR (KBr) *v*: 3323, 2928, 1672, 1570, 1549, 1501, 1362, 1288, 1140, 1024, 883; HRESIMS *m/z* calcd. for $C_{42}H_{28}N_8O_6S_2$ $[M + H]^+$ 805.1646, found: 805.1654.

4,6,16,18-Tetraaza-11,23-bis(N-formyl-02-hydroxyethylamino)-5,17-bis(methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (5l)

White solid; yield: 45.6%; m.p. 225.5–226.5 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) *δ*: 8.72 (t, $J = 5.2$ Hz, 2H), 7.63 (d, $J = 2.4$ Hz, 4H), 7.44 (t, $J = 2.4$ Hz, 2H), 5.84 (s, 2H), 4.85 (s, 2H), 3.46 (t, $J = 6.0$ Hz, 4H), 3.29 (q, $J = 6.0$ Hz, 4H), 2.54 (s, 6H); ^{13}C NMR (DMSO-d_6, 100 MHz) *δ*: 173.2, 171.9, 164.5, 153.5, 139.2, 118.3, 116.3, 86.3, 60.0, 42.9, 14.1; IR (KBr) *v*: 3416, 2928, 1655, 1572, 1551, 1439, 1361, 1292, 1167, 1144, 1026; HRESIMS *m/z* calcd. for $C_{28}H_{28}N_8O_6S_2$ $[M + H]^+$ 639.1326, found: 639.1334.

4,6,16,18-Tetraaza-11,23-bis(N-formyl-3-hydroxypropylamino)-5,17-bis(methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (5m)

White solid; yield: 17.2%; m.p. 171.5–172.5 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) *δ:* 8.58 (s, 2H), 7.59 (s, 4H), 7.45 (s, 2H), 5.94 (s, 2H), 4.47 (s, 2H), 3.42 (s, 4H), 3.28 (s, 4H), 2.55 (s, 6H), 1.63 (s, 4H); ^{13}C NMR (DMSO-d_6, 100 MHz) *δ:* 173.2, 171.9, 164.3, 153.7, 139.2, 118.0, 116.1, 86.5, 59.0, 37.2, 32.7, 14.1; IR (KBr) *v*: 3335, 2930, 1570, 1556, 1406, 1364, 1292, 1167, 1148, 1026, 900; HRESIMS *m/z* calcd. for $C_{30}H_{30}N_6O_8S_2$ $[M + Na]^+$ 689.1459, found: 689.1459.

4,6,16,18-Tetraaza-11,23-bis(N-formyl-5-hydroxypentylamino)-5,17-bis(methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (5n)

White solid; yield: 19.7%; m.p. 232.5–233.5 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) *δ:* 8.58 (s, 2H), 7.59 (s, 4H), 7.45 (s, 2H), 5.98 (s, 2H), 4.37 (s, 2H), 3.36 (s, 4H), 3.22 (s, 4H), 2.53 (d, $J = 14.2$ Hz, 6H), 1.47 (s, 8H), 1.30 (s, 4H); ^{13}C NMR (DMSO-d_6, 100 MHz) *δ:* 173.2, 171.9, 164.2, 153.7, 139.2, 117.9, 116.0, 86.5, 61.1, 32.7, 29.3, 23.5, 14.1; IR (KBr) *v*: 3344, 2933, 1643, 1570, 1406, 1365, 1289, 1140, 1025, 900, 804; HRESIMS *m/z* calcd. for $C_{34}H_{38}N_6O_8S_2$ $[M + Na]^+$ 745.2085, found: 745.2080.

4,6,16,18-Tetraaza-11,23-bis(N-formyl-diethanolamino)-5,17-bis(methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (5o)

White solid; yield: 21%; mp 228.5–229.5 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) *δ*: 7.35 (d, $J = 2.0$ Hz, 2H), 7.26 (d, $J = 2.0$ Hz, 4H), 5.45 (s, 2H), 4.81 (s, 4H), 3.57 (d, $J = 5.2$ Hz, 4H), 3.46 (dd, $J = 14.4$, 4.8 Hz, 8H), 3.23 (s, 4H), 2.54 (s, 6H); ^{13}C NMR (DMSO-d_6, 100 MHz) *δ*: 173.4, 171.8,

168.9, 153.1, 141.9, 118.5, 114.8, 85.6, 58.8, 52.1, 14.1; IR (KBr) *v*: 3441, 2926, 1676, 1572, 1551, 1501, 1362, 1290, 1140, 1022; HRESIMS *m/z* calcd. for $C_{32}H_{34}N_6O_{10}S_2$ $[M+H]^+$ 727.1851, found: 727.1867.

Pharmacology

Cell culture

The novel oxacalix[2]arene[2]pyrimidines (**5a**–**5o**) were screened to determine their preliminary anti-proliferative activity using four cultured cell lines: HeLa (human cervical cancer cell line), MCF7 (human breast cancer cell line), HepG2 (human hepatocarcinoma cell line), and A549 (human non-small cell lung cancer cell line). All the cell lines were obtained from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin. All were kept in standard conditions: 37 °C and 5% CO_2 in a humidified atmosphere. When 80% confluence was reached, cells were passaged with a solution containing 0.25% trypsin and 0.02% EDTA.

Cell viability assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to assess the in vitro cytotoxicity of the newly synthesized compounds (**5a**–**5o**) according to the methods described in previous studies (Liu et al. 2018; Chhajed et al. 2014; Arafa et al. 2014). In brief, the exponentially growing cells were seeded at a density of 3×10^3 cells per well in 96-well plates, and after 24 h of incubation at 37 °C in a 5% CO_2 incubator, the cells were exposed to the desired concentrations of target compounds for 72 h. Four hours prior to termination of the experiment, an MTT solution (20 µl of 5.0 mg/ml solution) was added to each well, and the plates were incubated at 37 °C under a humidified 5% CO_2. At the end of treatment, supernatants were aspirated from the wells, and the formazan crystals formed by cells were dissolved in DMSO (100 µl per well). Absorbance was measured at 550 nm using a 96-well microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). IC_{50} values were determined using GraphPad Prism 5 (Version 5.01, GraphPad Software, Inc., USA).

Cell apoptosis assay

We determined the apoptosis of MCF7 cells induced by representative compound **5l** using an Annexin V-FITC Apoptosis Detection Kit (Beyotime, Jiangsu, China) as described in previous studies (Zhang et al. 2018; Liu et al. 2016). MCF7 cells were seeded in six-well plates at a density of 1×10^5 cells/well and then treated with different concentrations of **5l** (0, 6, 12, and 24 µM) for 48 h. After treatment, cells were harvested, washed with cold PBS and incubated with annexin-V-FITC and PI in binding buffer for 15 min in the dark. Subsequently, a flow cytometer (Facs Canto II, BD, Inc., Franklin Lakes, NJ, USA) was used to analyze cell apoptosis (within 1 h after treatment). Cells undergoing early and late apoptosis were labeled with annexin-V and both annexin-V and PI, respectively.

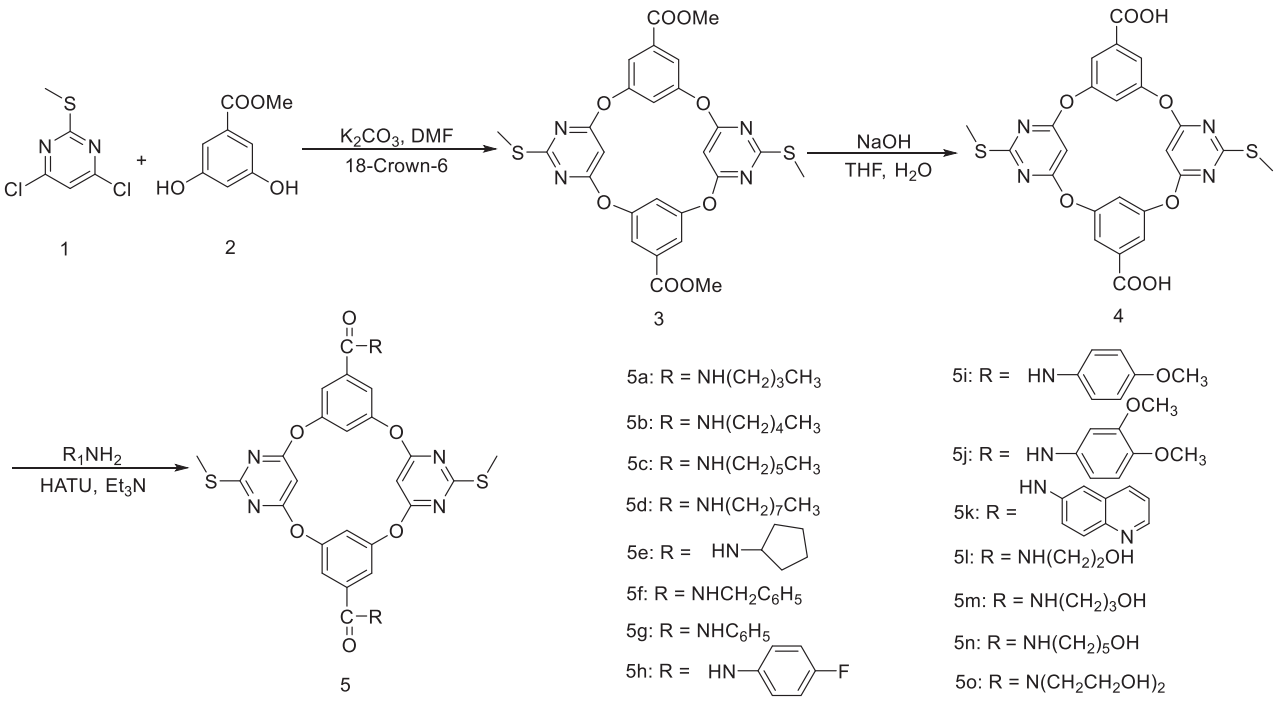
Statistical analysis

The results are representative of three independent experiments and are presented as the mean ± standard deviation (SD). Statistical analyses were carried out using the SPSS 16.0 software package, and the differences between the groups were assessed with a one way analysis of variance (ANOVA) followed by Dunnett's test. We considered $p < 0.05$ to be statistically significant.

Results and discussion

Chemistry

A convenient synthetic route for the preparation of oxacalix[2]arene[2]pyrimidines with various substituents is shown in Scheme 1. The oxacalix[2]arene[2]pyrimidine core **3** was directly obtained via a one-pot condensation of **1** (4,6-dichloro-2-methylthiopyrimidine) and **2** (methyl 3,5-dihydroxybenzoate) in the presence of potassium carbonate and 18-crown-6 in dimethylformamide (DMF) at 75 °C. Initially, we tried to synthesize the target compounds **5a**–**5o** through the direct ammonolysis reaction of 4,6,16,18-Tetraaza-11,23-bis(methylformate)-5,17-bis(methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (**3**) with amines. However, this reaction only provides a trace amount of target compounds when the amines are hydroxylamines, and when the amines are aromatic amines or aliphatic amines, the reaction does not provide any target compounds. Hence, we chose to synthesize **5a**–**5o** via condensation of 4,6,16,18-Tetraaza-11,23-bis(formicacid)-5,17-bis(methylsulfanyl)-2,8,14,20-tetraoxacalix[4]arene (**4**) with the corresponding amines. Intermediate **3** was hydrolyzed to **4** via treatment with sodium hydroxide in THF/H_2O, and ultrasonic irradiation was introduced to this reaction to reduce the reaction time to almost 4 h. A moderate yield of the target compounds **5a**–**5o** was obtained from HATU catalysis in DMF at room temperature.



Scheme 1 Synthesis of oxacalix[2]arene[2]pyrimidines **5a–5o**

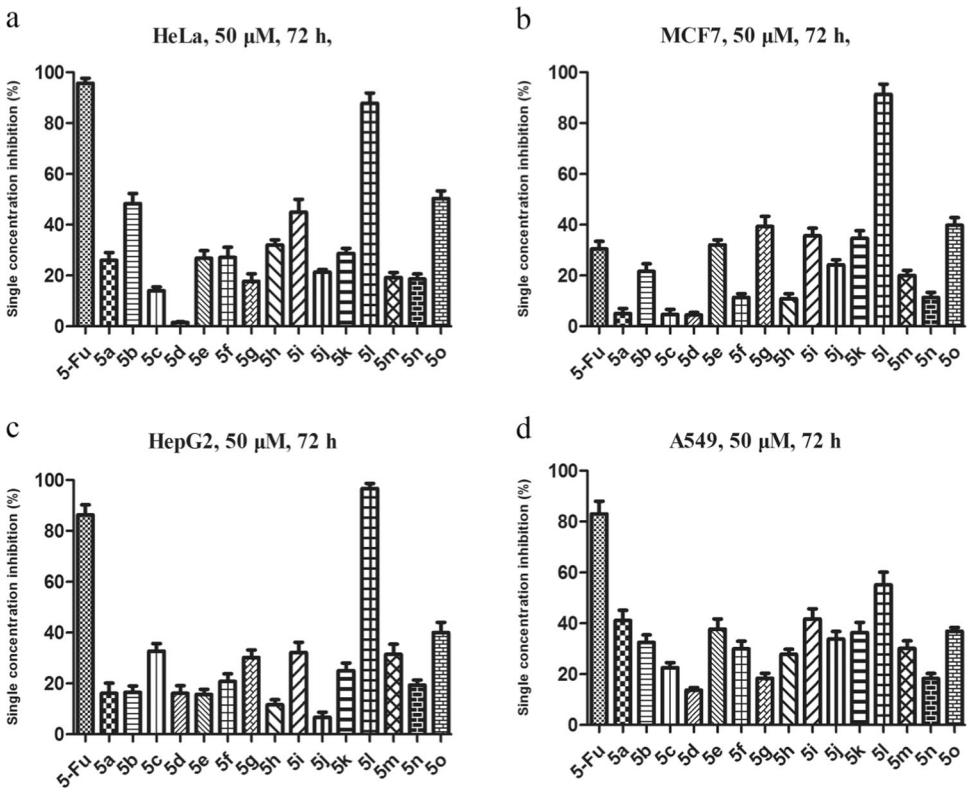
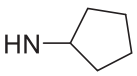
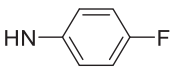
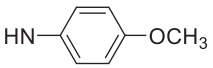
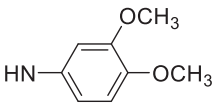
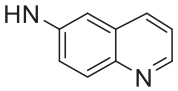


Fig. 2 Single concentration inhibition rates of compounds **5a–5o** on HeLa **a**, MCF7 **b**, HepG2 **c** and A549 **d** cells

In vitro anticancer evaluations

All the synthesized oxacalix[2]arene[2]pyrimidine derivatives, **5a–5o**, underwent preliminary screening to determine their anticancer activities. An MTT assay was performed for human cervical cancer cells (HeLa, Fig. 2a), human breast cancer cells (MCF7, Fig. 2b), human hepatocarcinoma cells (HepG2, Fig. 2c), and human non-small cell lung cancer

Table 1 SAR of oxacalix[2]arene[2]pyrimidine derivatives **5a–5o**

Compounds	R	Single concentration inhibition (%)[a]				Log*P*[b]
		HeLa	MCF7	HepG2	A549	
5a	$NH(CH_2)_3CH_3$	26.01	5.00	16.16	41.12	8.84
5b	$NH(CH_2)_4CH_3$	48.41	21.64	16.50	32.49	9.81
5c	$NH(CH_2)_5CH_3$	14.00	4.70	32.70	22.57	10.77
5d	$NH(CH_2)_7CH_3$	1.50	4.48	16.14	13.74	12.70
5e		26.83	32.00	15.74	37.72	7.76
5f	$NHCH_2C_6H_5$	27.12	11.39	20.78	29.93	9.25
5g	NHC_6H_5	17.71	39.33	30.18	18.39	9.31
5h		32.00	10.88	11.59	27.76	9.84
5i		45.08	35.63	32.18	41.69	9.49
5j		21.38	24.20	6.67	33.79	9.43
5k		28.71	34.65	25.00	36.38	10.20
5l	$NH(CH_2)_2OH$	87.89	91.34	96.67	55.13	4.59
5m	$NH(CH_2)_3OH$	19.17	20.00	31.49	30.10	5.56
5n	$NH(CH_2)_5OH$	18.64	11.38	19.00	18.26	7.49
5o	$N(CH_2CH_2OH)_2$	50.37	39.78	40.00	36.86	3.60
5-FU		95.70	30.47	86.29	83.07	-0.36

[a]The concentration of the oxacalix[2]arene[2]pyrimidines **5a–5o** was 50 μM

[b]These values were calculated using the online software Molsoft

cells (A549, Fig. 2d). Cell viability was evaluated after 72 h of incubation in the presence of the target compounds at a concentration of 50 μM, and 5-Fluorouracil (**5-FU**) was served as a positive control under similar conditions. As illustrated in Fig. 2, oxacalix[2]arene[2]pyrimidines with diverse amide substituents exhibited varying degrees of anticancer activity. This result might be related to the differences in lipophilicity between the synthesized compounds, because ligand lipophilicity can impact ligand–protein affinity and various important properties, such as absorption, distribution, metabolism, and excretion (Johnson et al. 2018; Tantry et al. 2017). The octanol/water partition coefficients (Log*P*) were calculated using the online software Molsoft (http://www.molsoft.com/mprop/), and all the preliminary screening results and predicted Log*P* values are summarized in Table 1.

As shown in Table 1, compounds **5a–5e**, which featured aliphatic amides, exhibited moderate anticancer activity against all four cancer cell lines. Of those five compounds, **5d** had the lowest anti-proliferative efficacy, indicating that excessive chain length and high lipophilicity are detrimental to anticancer activity. Compounds **5f–5k**, which featured aromatic amide groups, exhibited similar lipophilicity and moderate cytotoxicity for all cell lines. Of the compounds **5f–5k**, the activity of *p*-methoxyaniline derivative **5i** was more promising than that of the other derivatives; the inhibition rates of HeLa and A549 cells were 45.08% and 41.69%, respectively. Compound **5l**, which contains a short-chained hydrophilic ethanolamine moiety, exhibited the strongest inhibitory activity against HeLa, MCF7, HepG2, and A549 among the synthesized compounds. In contrast to **5-FU**, **5l** exhibited superior anti-proliferative efficacy for MCF7 and HepG2, and

almost entirely inhibited those cancer cell lines at a concentration of 50 μM (MCF7, 91.34%; HepG2, 96.67%). The anticancer activity of **5l** against HeLa was similar to that of **5-FU**, with an inhibition rate of 87.89%. Moreover, consistent with the inferior cytotoxicity of all oxacalix[2]arene[2]pyrimidines for A549, compound **5l** induced cell death in only 55.3% of A549 cells, indicating that these compounds are not suitable for lung cancer treatment. The optimal antitumor activity of the hydroxylamine derivative **5l** could be attributed to its appropriate lipophilicity, and increased lipophilicity was found to greatly attenuate antitumor activity (as seen in compounds **5m** and **5n**). To our surprise, the more hydrophilic compound **5o** displayed relatively poor inhibitory activity compared to **5l**, suggesting that the NH group of the amide plays a critical role in antitumor activity. Overall, the structure–activity relationship (SAR) studies revealed that introduction of a hydrophilic and short-chained secondary amide group could improve the activity of oxacalix[2]arene[2]pyrimidines.

Based on the above results, we conducted a follow-up biological evaluation of **5l** on HeLa, HepG2, and MCF7 cells. The IC_{50} values representing the concentration required for a 50% decrease in cell growth were calculated and are presented in Table 2. Compound **5l** exhibited considerable activity against HeLa, HepG2, and MCF7 cells, with IC_{50} values of 20.30, 12.37, and 13.18 μM, respectively. The antiproliferative effect of **5l** on HepG2 and MCF7 cells was superior to that of **5-FU**, especially for MCF7, for which **51** exhibited nearly six times the inhibitory activity of **5-FU** (Table 2).

Cell apoptosis assay

Since the antitumor activity of calixarene was reported to be related to apoptosis, we hypothesized that the cytotoxic effect of **5l** might be associated with apoptosis (An et al. 2016; Addepalli et al. 2018). To verify this hypothesis, HepG2 cells were exposed to diverse concentrations of **5l** (0, 6, 12, 24 μM) for 48 h, and analyzed by Annexin V-FITC/PI staining followed by flow cytometry analysis to quantitatively measure apoptosis. As shown in Fig. 3, the apoptotic rates of HepG2 cells treated with 0, 6, 12, and 24 μM of **5l** for 48 h were 0.13%, 33.80%, 80.14%, and 90.57%, respectively. The percentage of apoptotic cells

Table 2 IC_{50} (μM) of **5l** and **5-FU** on HeLa, HepG2, and MCF7 cell lines

Compounds	IC_{50} (μM)[a]		
	HeLa	HepG2	MCF7
5l	20.30 ± 2.03	12.37 ± 1.99	13.18 ± 1.87
5-FU	10.83 ± 0.18	22.23 ± 2.85	73.94 ± 5.15

[a]IC_{50} is the concentration that inhibits 50% of cell growth

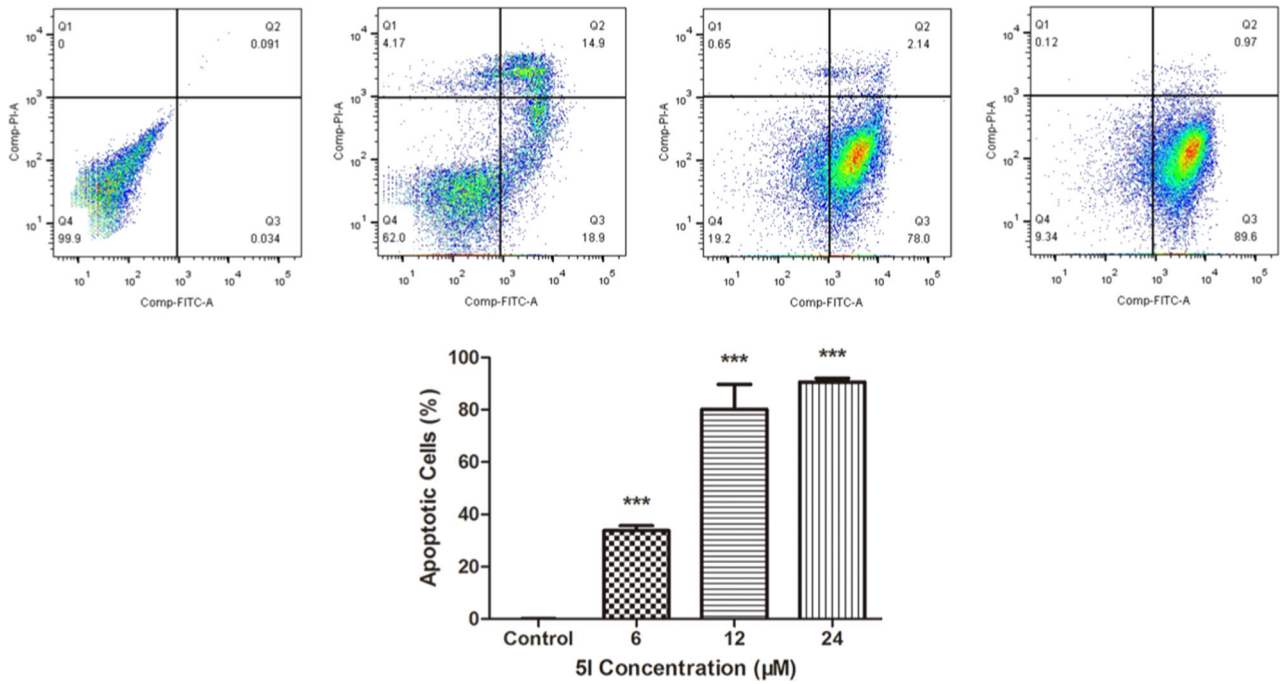


Fig. 3 Pro-apoptotic effect of compound **5l** on HepG2 cells. HepG2 cells were treated with 0, 6, 12, and 24 μM of **51** for 48 h. Then, the cells were stained with Annexin V-FITC/PI and underwent flow cytometry analysis. $^{***}p < 0.001$ compared with the control group

significantly increased with increasing concentrations of **5l**, indicating that the anticancer effect of **5l** might be involved in the induction of apoptosis.

Conclusion

In summary, we designed and synthesized a series of novel oxacalix[2]arene[2]pyrimidine derivatives (**5a**–**5o**). All these compounds were initially screened for single concentration inhibition on four different human cancer cell lines (HeLa, MCF7, HepG2, and A549) at a concentration of 50 μM. The activity of **5l** against HeLa, MCF7, and HepG2 cells was comparable to the positive control **5-FU**. The IC_{50} values of compound **5l** for the HeLa, HepG2, and MCF7 cell lines were 20.30, 12.37, and 13.18 μM, respectively. A cell apoptosis assay indicated that the antiproliferative activity of **5l** might be related to apoptosis. These promising results support the potential anticancer efficacy of oxacalix[2]arene[2]pyrimidines, and further structure modification as well as biological study is still carried out in our lab.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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