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The synthesis and anticancer activities of chiral epoxy-substituted chromone analogs

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A B S T R A C T

Human DNA topoisomerases (topos) have been recognized as a good target molecule for the development of anticancer drugs because they play an important role in solving DNA topological problems caused by DNA strand separation during replication and transcription. In this study, we designed and synthesized 11 novel chromone backbone compounds possessing epoxy and halohydrin substituents with chirality. In the topos inhibition test, compounds **2**, **9**, **10**, and **11** showed comparable topo I inhibitory activity at concentration of 100 μM compared to camptothecin, and all of the synthesized compounds showed moderate topo IIα inhibitory activity. Among them, compounds **9**, **10** and **11** were more potent than the others in both topo I and IIα inhibitory activity. Compound **11** showed the most potent cell antiproliferative activity against all tested cancer cell lines with particularly strong inhibition (an IC_{50} of 0.04 μM) of K562 myelogenous leukemia cancer cell proliferation. In the brief structure-activity relationship analysis, there was no clear correlation between stereochemistry and topos inhibitory and cytotoxic activity. 5(*R*),7(*S*)-bisepoxy-substituted compound **11** was the most potent DNA cross-linker and induced G2/M arrest in a cell cycle assay in a dose- and time-dependent manner. After the treatment time period induced apoptosis in K562 cells without increasing G2/M-phase cells. Overall, compound **11** showed good consistent inhibitory biological activity related to cancer cell proliferation.

1. Introduction

Human DNA topoisomerases (topos) have been recognized as a good target molecule for the development of anticancer drugs because they play an important role in solving DNA topological problems caused by DNA strand separation during replication and transcription. Human DNA topos consisting of topo I and topo II change the topology of intertwined DNA by making a transient breaking of the DNA single-strand by topo I or double-strands by topo II, followed by religation of the cleaved strand(s) [1,2]. Topo inhibitors account for a considerable proportion of the 150 anticancer therapeutic drugs approved by the US Food and Drug Administration during the period from 1949 to 2014 [3]. Topo-inhibiting drugs currently in clinical use are doxorubicin (Adriamycin), etoposide, teniposide, irinotecan, topotecan hydrochloride, idarubicin, daunorubicin, valrubicin, and epirubicin [3]. A number of clinical trials have been studied for topo inhibitor alone such as LMP400 and LMP776 (indenoisoquinoline type topo I inhibitors, ClinicalTrials.gov identifier: NCT01051635) or in combination with diverse chemotherapeutics and targeted drugs, such as apatinib (a vascular endothelial growth factor receptor-2 tyrosine kinase inhibitor, ClinicalTrials.gov identifier: NCT03100955), oxaliplatin (a platinum-based antineoplastic agent, ClinicalTrials.gov identifier: NCT01523457, NCT01523457), and selinexor (a selective inhibitor of nuclear export (SINE), ClinicalTrials.gov identifier: NCT02283359), VX-970 (a selective ataxia telangiectasia and rad3-related kinase inhibitor, ClinicalTrials.gov identifier: NCT0259593) for the treatment of advanced small cell lung cancer, adenocarcinoma of the stomach and distal esophagus, and metastatic pancreatic cancer [4–8]. Although many topo-targeting drugs are already in clinical use, medicinal chemists are constantly interested in developing novel topo-inhibiting drugs to overcome the drawbacks of the currently marketed ones, such as the lack of chemical stability and topo enzyme isotype specificity, and the induction of resistance [9–13]. During the past few years, we have tried to design and synthesize potentially drug-like compounds including epoxy- and thioepoxy-substituted xanthone and benzoxanthone analogs that can inhibit DNA synthesis by topos inhibition and/or by DNA alkylation [14–23].

Compounds containing a chromone and hydroxynaphthalene core have shown a variety of biological activities such as the dimerization-inhibitory activity of heat-shock protein 27, leading to inducing lung cancer susceptibility to radiation therapy and reducing radiation-induced lung inflammation [24,25], cholinesterase inhibitory activity [26], radical scavenging ability with neuroprotective effect in the dopaminergic neurodegenerative zebrafish model [27], an anti-obesity effect through the inhibition of glycogen synthase kinase-3 [28], anticancer activity by the inhibition of phosphoinositide 3- kinase [29],

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Table 1
The topo I and IIα inhibitory activity of the synthesized compounds.

Compounds	Topo I (% Inhibition)		Topo IIα (% Inhibition)	
	100 μM	20 μM	100 μM	20 μM
Camptothecin	67.0	49.2	–	–
Etoposide	–	–	62.1	43.3
1	0	0	16.9	8.9
2	67.4	29.6	30.0	19.6
3	0	0	36.9	27.4
4	0	0	33.4	21.3
5	0	0	33.2	22.5
6	1.1	0	35.2	20.1
7	1.9	0	36.7	29.7
8	9.7	1.7	23.2	18.0
9	62.2	23.8	51.8	25.8
10	67.3	38.0	59.3	20.1
11	61.2	34.3	47.5	16.1

and anti-hepatocarcinogenic activity by activating the small heterodimer partner [30]. Chromone analogs are also known to be effective topo I inhibitors but did not shown efficient anticancer activity against the MCF-7, KB, and NCI-H187 cancer cell lines [31] (see Table 1).

As part of the continuous effort to develop compounds that exhibit anticancer effects by inhibiting the DNA synthesis of cancer cells, we designed and synthesized 11 novel compounds with an epoxy-substituted chromone backbone (Fig. 1). In this series, we also tried to understand the effect of stereochemistry on their pharmacological activity at the epoxy-ring site. Their ability to alkylate DNA and inhibit topo enzymes and cancer cell growth were also evaluated.

2. Results and discussion

2.1. Chemistry

The coupling reaction of 5,7-dihydroxy-2-methyl-4*H*-chromen-4-one (prepared according to a previously reported method [32]) and epichlorohydrin (4–5 equiv) was conducted under basic K_2CO_3 (2–2.5 equiv) condition in acetone (**1** and **2**). In a ^{1}H NMR spectrum, compound **1** showed two doublet of doublet peaks at δ 2.81 and 2.97 and one multiplet at δ 3.36–3.39, corresponding to the two methylene protons and the methine proton of the epoxide ring, respectively. Generally, the pharmacological activity of compounds possessing a stereocenter can be different due to their chirality, so with this in mind, we also tried to synthesize stereochemically pure isomers to test and compare the anticancer activity between the stereoisomers. For the enantiomeric pure stereo isomer synthesis, (*R*)- or (*S*)-epichlorohydrin was used on compounds **4**, **5**, **7** and **8**. The epoxy ring-opening reaction was accomplished in 3 M-HCl/EtOAc to give the desired halohydrin compounds **3**, **6** and **9** (see Scheme 1).

2.2. The topo I and IIα inhibitory activity of the compounds

We evaluated the newly synthesized 11 chromone derivatives for topo I and IIα inhibitory activity, the results of which are shown in Fig. 2 and Table 2. We measured the degree of conversion of supercoiled plasmid DNA to relaxed DNA by topo I or IIα in the presence of our synthesized compounds. Compounds **2**, **9**, **10**, and **11** showed comparable topo I inhibitory activity compared to camptothecin (a well-known topo I inhibitor) at concentration of 100 μM, but their activity was slightly lower at 20 μM. All of the synthesized compounds showed topo IIα inhibitory activity at concentrations of 20 and 100 μM, although they were weaker than etoposide (a well-known topo II inhibitor clinically used as an anticancer drug currently). Compounds **9**,

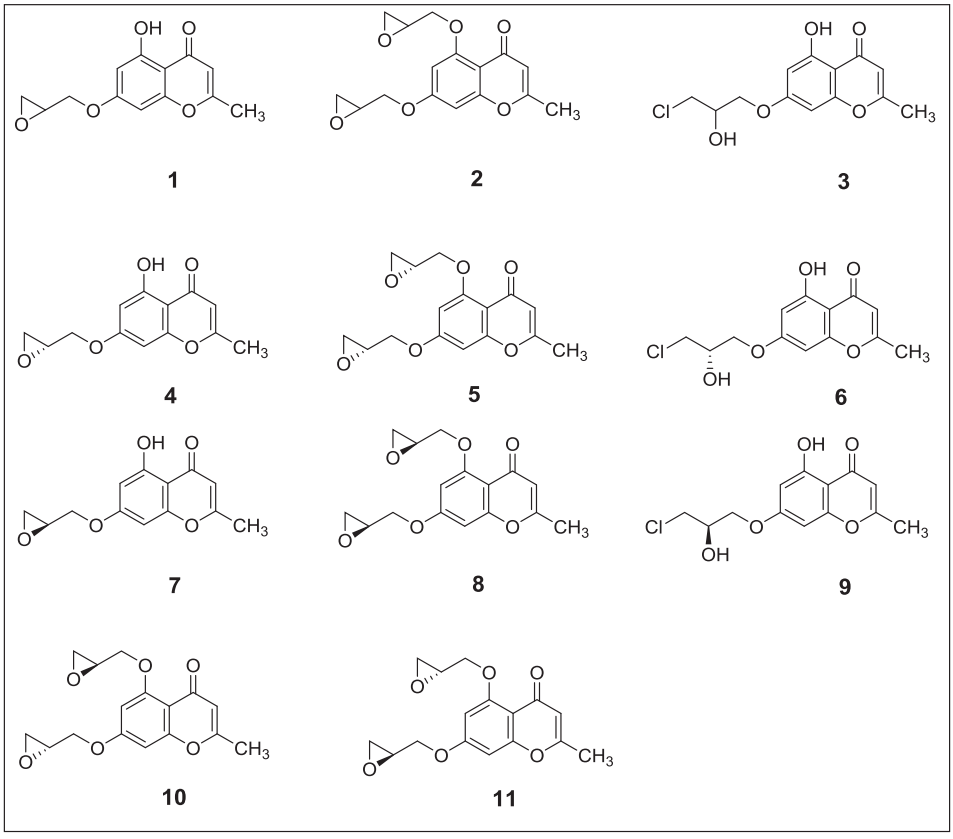
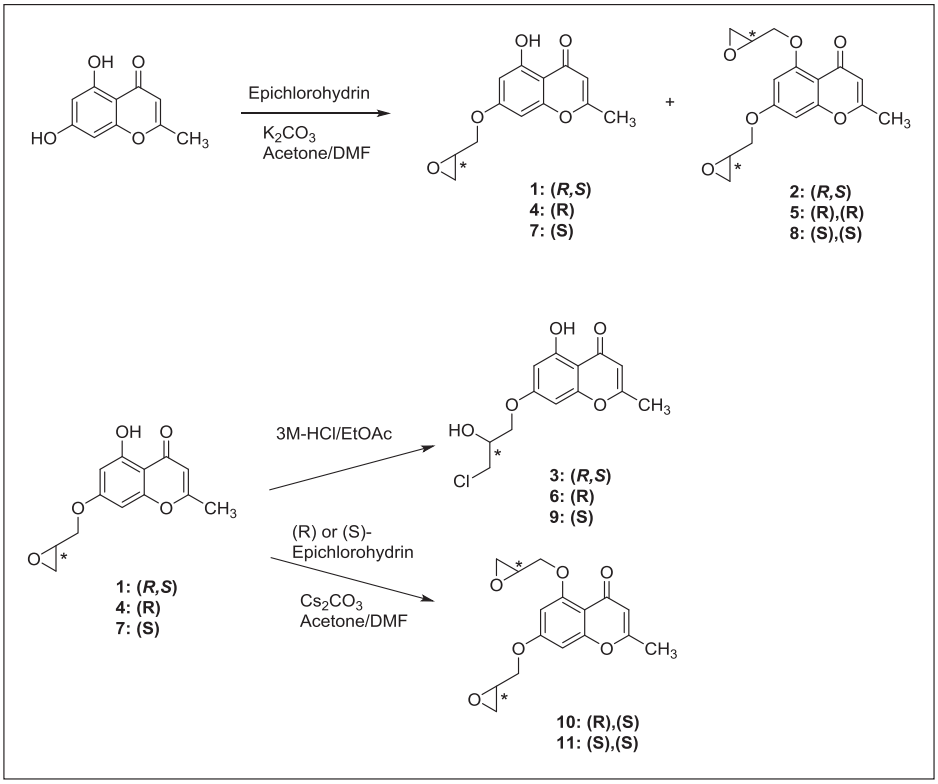


Fig. 1. The structures of the 11 synthesized compounds.



Scheme 1. The synthetic routes for the compounds.

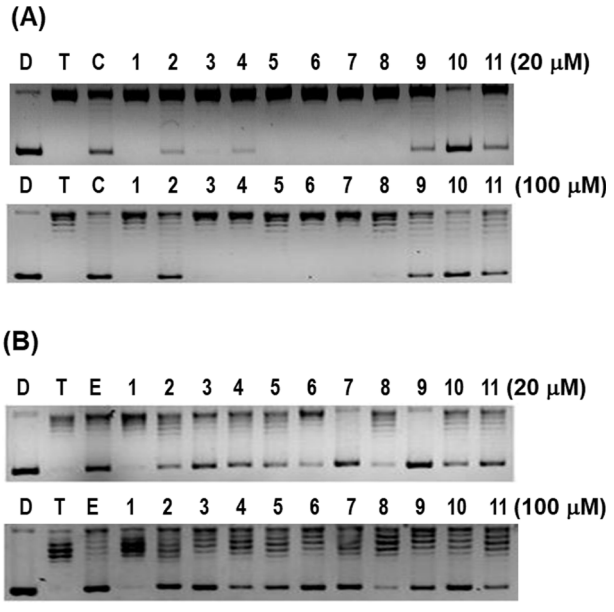


Fig. 2. Topo I and IIα inhibitory activities of the compounds. (A) Lane D: pBR322 DNA only; Lane T: pBR322 DNA + topoisomerase I; Lane C: pBR322 DNA + topoisomerase I + camptothecin; Lane 1–11: pBR322 DNA + topoisomerase I + compounds 1–11 (in that order). (B) Lane D: pBR322 DNA only; Lane T: pBR322 DNA + topoisomerase IIα; Lane E: pBR322 DNA + topoisomerase IIα + etoposide; Lane 1–11: pBR322 DNA + topoisomerase IIα + each of compounds 1–11 (in that order).

10, and **11** inhibited topo IIα at 100 μM with moderate intensities of 51.8%, 59.3%, and 47.5%, respectively, compared to 62.1% for etoposide. Compounds **2**, **3**, **4**, **5**, **6**, and **7** inhibited topo IIα in the range 30.0–36.9% and compounds **1** and **8** inhibited topo IIα by 16.9% and 23.2%, respectively. Among the compounds, **10** most strongly inhibited topo I and topo IIα. In the topoisomerase inhibitory activity, all of the mono epoxy substituted analogs (**1**, **4** and **7**) had no effect on topo I functionality, but showed mild topo IIα inhibitory activity. Among the epoxide ring-opened halohydrin compounds **3**, **6** and **9**, compound **9** derived from the (*S*)-epoxy isomer **7** was around 1.5-fold more active than the others for both topo I and topo IIα inhibitory activity. Among the bisepoxy substituted compounds **2**, **10,** and **11** showed better inhibitory activity than the others (**5** and **8**). Interestingly, they possessed an epoxide ring stereochemically opposed at the 5 and 7 positions, and we wonder whether the steric factors of the two substituents were influential on the topoisomerase I inhibitory activity.

2.3. The cell antiproliferative effect of the compounds

An evaluation of the cell antiproliferative activity was performed on five different human cell lines: MCF7 (human breast adenocarcinoma), HeLa (human cervix adenocarcinoma), DU145 (human prostate cancer), HCT15 (human colorectal adenocarcinoma), and K562 (human myelogenous leukemia) by measuring the mitochondrial dehydrogenase activity. The IC_{50} values (μM) of the prepared compounds against these cell lines are listed in Table 2. Adriamycin, camptothecin, and etoposide were used as positive controls. Of the compounds exhibiting topo I and topo IIα dual inhibition (**2** and **9**–**11**), **11** showed the most potent cell antiproliferative activity against all tested cancer cell lines with particularly strong inhibition (an IC_{50} of 0.04 μM) of K562 myelogenous leukemia cancer cell proliferation. Moreover, it exhibited much more potent cell antiproliferative activity in K562 cells compared to all of positive controls. Compounds containing an oxiranylmethoxy group (**1**, **4**, and **7**) showed mild cell antiproliferative activity against all of the tested cancer cell lines, while those with a 3-chloro-2-hydroxypropoxy group (**3**, **6**, and **9**) showed even weaker cell antiproliferative activity. Although there was some discrepancy due to the different types of cancer cell lines in the cytotoxicity test, monoepoxy-substituted analogs **1**, **4** and **7** inhibited cancer cell growth to almost the same degree, and epoxide ring-opened halohydrin

Table 2
Cell anti-proliferative activity IC_{50} values (μM) of the synthesized compounds against various cancer cells lines.

Comp/cell	MCF7	HeLa	DU145	HCT15	K562
Adriamycin	1.05 ± 0.09	5.46 ± 0.11	3.76 ± 0.25	5.83 ± 0.18	1.71 ± 0.21
Etoposide	2.04 ± 0.24	2.17 ± 0.07	2.04 ± 0.24	6.75 ± 0.00	4.67 ± 0.40
Camptothecin	2.80 ± 0.05	6.00 ± 0.00	1.99 ± 0.91	1.59 ± 0.15	2.69 ± 0.15
1	13.6 ± 1.83	17.7 ± 1.72	20.9 ± 0.53	18.3 ± 0.60	14.3 ± 2.67
2	6.5 ± 1.06	5.4 ± 0.96	7.3 ± 0.31	12.1 ± 0.74	6.3 ± 2.53
3	43.63 ± 4.08	24.02 ± 1.65	43.41 ± 0.86	2.51 ± 0.01	52.82 ± 5.55
4	22.92 ± 2.26	16.69 ± 0.25	15.16 ± 0.88	27.18 ± 2.40	9.74 ± 0.30
5	2.20 ± 0.41	11.41 ± 0.15	15.57 ± 0.34	2.29 ± 0.13	7.86 ± 0.26
6	26.06 ± 0.05	30.41 ± 0.03	53.74 ± 0.59	42.20 ± 2.36	35.93 ± 1.79
7	36.21 ± 2.73	11.92 ± 0.83	7.58 ± 0.38	17.53 ± 0.47	8.03 ± 0.81
8	46.07 ± 0.97	21.65 ± 3.04	24.52 ± 1.35	39.52 ± 2.20	19.49 ± 1.92
9	37.11 ± 3.35	47.03 ± 1.78	42.14 ± 4.68	47.46 ± 4.69	20.24 ± 10.02
10	13.87 ± 0.79	10.95 ± 2.47	20.38 ± 0.92	12.08 ± 0.04	1.49 ± 0.38
11	1.88 ± 0.05	1.77 ± 0.32	6.28 ± 0.46	9.59 ± 0.37	0.04 ± 0.03

Each data item is the mean ± standard deviation (S.D.) of the experiments performed in triplicate.

compounds **3**, **6** and **9** showed similar results. 5(*R*),7(*S*) bisepoxy-substituted compound **11** and racemic compound **2** showed much more potent cytotoxicity than the other bisepoxy-substituted compounds. Especially, **11** showed stronger cytotoxic activity than **2** for all of the tested cancer cell lines and particularly strong inhibition (an IC_{50} of 0.04 μM) for K562 cancer cell proliferation.

2.4. The DNA interstrand cross-linking activity of compounds

Based on our previous report about the ability of 1,3-bisepoxyxanthone to form a cross-linked DNA duplex due to its electrophilic nature by bisepoxy groups [18], we first checked the cross-linking activity of compounds **1**, **2**, and **3**, which are the mixture of stereoisomers. Compound **2** only possessing two epoxy groups showed strong DNA cross-linking activity (Fig. 3A), but the others possessing an epoxy group or a 3-chloro-2-hydroxypropoxy group were inactive to nucleophiles. In our previous study we observed that certain compounds did not produce any DNA cross-linked adducts despite having bisepoxy groups, which might have been caused by the intercalating position in the bisepoxy-benzoxanthone derivatives [18]. Therefore, we further evaluated bisepoxy-substituted compounds **2**, **5**, **8**, **10**, and **11** to determine any stereo-specificity in the cross-linking activity. Compound **11** (with 5(*R*),7(*S*)-bisepoxy substitution) was the most potent among the isomers, although the extent of the DNA cross-linked adduct caused by **11** was similar to that of **2** (Fig. 3B and C).

2.5. Compound 11 induced a G2/M arrest in the cell cycle of K562

Since compound **11** showed comparable topo I and IIα inhibitory activity compared to camptothecin and etoposide as well as the most potent DNA cross-linking activity and K562 cell antiproliferation, we evaluated its effects on K562 cells. In addition, the cell antiproliferative effect is considered to be a highly effective strategy for the treatment of rapidly proliferating tumors, therefore the effect of compound **11** on the cell cycle was assessed using FACS by staining with PI in K562 cells. Treatment with compound **11** for 16 h increased the number of cells in the G2/M phase at a very low concentration of 0.1 μM, which peaked at a treatment of 0.5 μM. At concentrations higher than 0.5 μM, the cells in the G2/M phase did not increase any further, but the proportion of sub-G1 cells increased (Fig. 4A). In addition, the increase in cells in the G2/

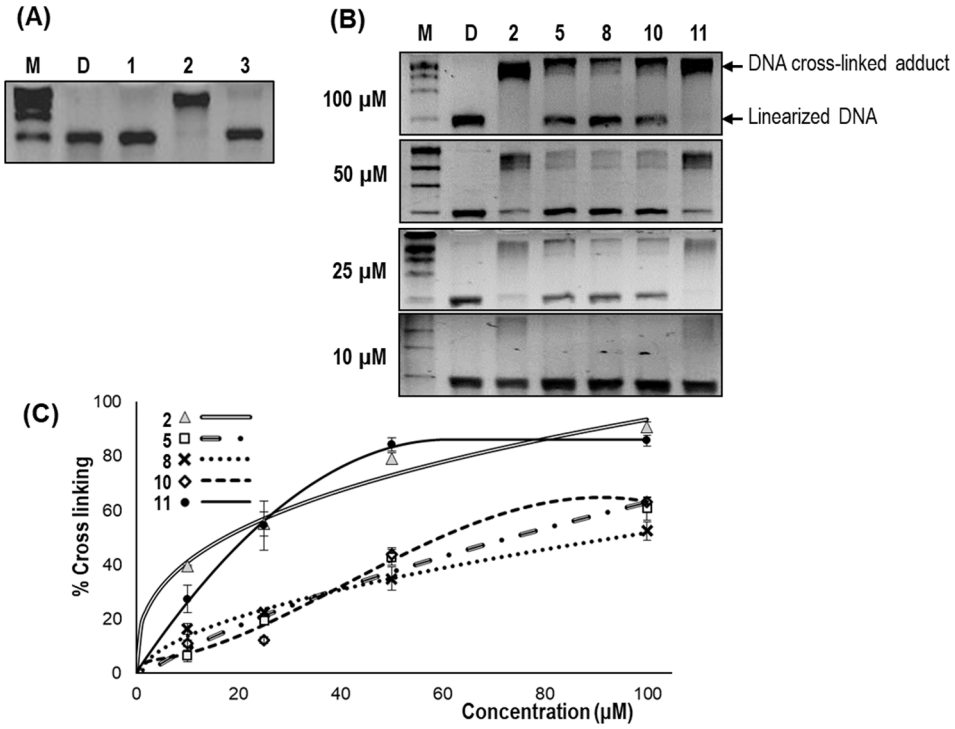


Fig. 3. DNA interstrand cross-linking activity of the selected compounds. (A) Lane M: DNA *Hin*dIII marker; Lane D: linearized pBR322 plasmid DNA only; Lanes 1, 2, and 3: linearized pBR322 + each individual compound at a final concentration of 100 μM. (B) Lanes 2, 5, 8, 10, and 11: linearized pBR322 + each individual compound at various concentrations of 10–100 μM (a representative image from 3 different experiments). (C) A graph plot of the average values obtained from the three experiments in (B). The error bars represent standard deviations.

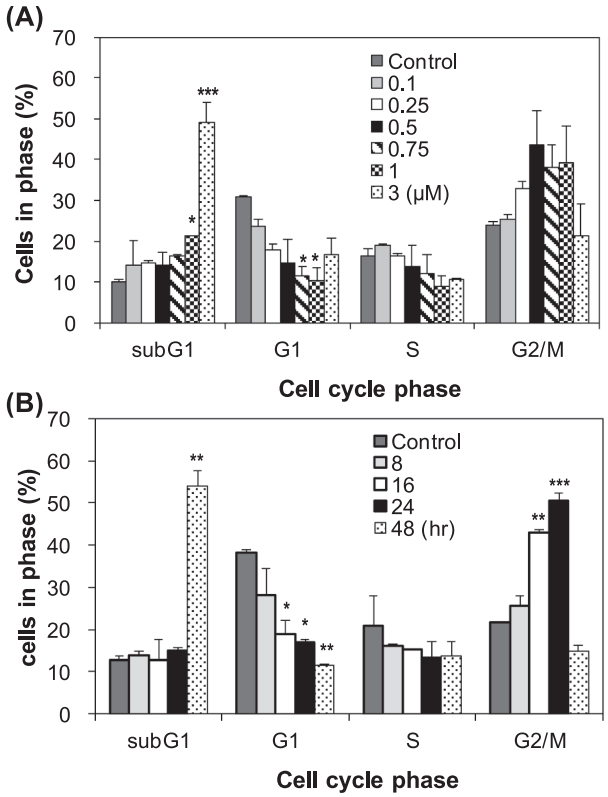


Fig. 4. The cell cycle analysis of K562 cells treated with compound 11. K562 cells were treated with 0, 0.1, 0.25, 0.5, 0.75, 1 or 3 μM of compound 11 for 16 h (A), and treated with 0.5 μM for 0, 8, 16, 24, or 48 h. The cell cycle distribution was then evaluated by using a flow cytometric DNA content analysis. Compound 11 clearly increased the G2/M fraction in a dose- and time-dependent manner. Columns and error bars indicate mean ± S.D. The data were analyzed using Student's t-tests and one-way ANOVA: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ indicate a significant difference compared to the control value.

M phase started to appear after 8 h of treatment with 0.5 μM compound **11**, which was augmented with increasing treatment time up to 24 h (Fig. 4B). Compound **11** induced G2/M arrest in a dose- and time-dependent manner and after a period of treatment time and induced apoptosis without increasing the G2/M phase cells.

3. Conclusions

Topos have been considered as a good therapeutic target in anticancer drug development. To overcome the drawbacks of currently marketed drugs, much effort has been invested to find better ones that manipulate cell proliferation. In this study, we designed and synthesized 11 novel chromone backbone compounds possessing epoxy and halohydrin substituents with chirality. In the topos inhibition testing, compounds **2**, **9**, **10**, and **11** showed comparable topo I inhibitory activity at a concentration of 100 μM compared to camptothecin and all of the synthesized compounds showed topo IIα inhibitory activity albeit weaker than etoposide at concentrations of 20 and 100 μM. Especially, compounds **9**, **10** and **11** were much more potent than other compounds in terms of both topo I and topo IIα inhibition. Of the compounds exhibiting topo I and topo IIα dual inhibition (**2** and **9**–**11**), compound **11** showed the most potent cell antiproliferative activity against all tested cancer cell lines, with particularly strong inhibition (an IC_{50} of 0.04 μM) for K562 cancer cell proliferation. In the brief structure-activity relationship analysis, there was no clear correlation between compounds' stereochemistry and their topos inhibitory and cytotoxic activity. 5(*R*),7(*S*) bisepoxy-substituted compound **11** also showed the most potent DNA cross-linker. The effect of compound **11** on the cell cycle was assessed using FACS by staining with PI in K562 cells; it induced G2/M arrest in a dose- and time-dependent manner and after the period of treatment time, induced apoptosis with G2/M phase arrest. Overall, compound **11** showed good consistent inhibition of the biological activities related to cancer cell proliferation.

4. Experimental

4.1. General experimental procedures for chemical synthesis

The solvents and reagents used were of the highest commercial grade available and used as received. The thin-layer chromatography plates were Kieselgel 60 F254 (art A715, Merck). Silica gel 60 (0.040–0.063 mm ASTM, Merck) was used for column chromatography. The purity was assessed using a high-performance chromatography (HPLC; Shimadzu LC-20AD) analysis under the following conditions; a SunFire C18 column (4.6 × 150 mm, 5 μm), a mobile phase of isocratic 50% acetonitrile in water (0.1%-formic acid) over 10 min, a flow rate of 1.0 mL/min; detection, and Shimadzu Spd-M20A diode array detector. The purities of the compounds are reported as percentage (%). ^{1}H- and ^{13}C NMR spectra were recorded on a Varian NMR AS 400 MHz instrument. Chemical shifts (δ) are in parts per million (ppm) relative to tetramethylsilane as an internal standard, and the coupling constants (*J* values) are in Hertz. Mass spectral investigations were performed on a Shimadzu GC/MS-QP2010 (Japan) instrument. The melting points were measured on a Gallenkamp Melting Point Apparatus without correction.

4.1.1. The synthesis of 5-hydroxy-2-methyl-7-(oxiran-2-ylmethoxy)-4H-chromen-4-one (1) and 2-methyl-5,7-bis(oxiran-2-ylmethoxy)-4H-chromen-4-one (2)

Epichlorohydrin (1.69 g, 18.20 mmol) was added to a reaction mixture of 5,7-dihydroxy-2-methyl-4*H*-chromen-4-one (0.69 g, 3.64 mmol) and K_2CO_3 (1.01 g, 7.28 mmol) in dimethylformamide (DMF)/acetone (9 mL/3 mL). The reaction mixture was stirred at ~80 °C for 14 h and then cooled to room temperature. After adding water, the reaction mixture was extracted with ethyl acetate. The organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and then the residue was purified using silica gel column chromatography (eluent: ethyl acetate:*n*-hexane = 1:1 → methanol (MeOH): chloroform = 5:95) to attain compounds **1** (0.24 g, 26.7%) as an ivory-colored solid and **2** (0.33 g, 29.5%) as a brown syrup.

Compound 1: R_f 0.51 (ethyl acetate:*n*-hexane = 1:1); mp: 132–133 °C; HPLC: R_T 4.77 min (purity; 99.6%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.37 (s, 3H), 2.79 (S, 1H), 2.95 (S, 1H), 3.38 (S, 1H), 3.98 (dd, *J* = 10.8, 5.2 Hz, 1H), 4.31 (d, *J* = 10.8 Hz, 1H), 6.05 (S, 1H), 6.36 (S, 1H), 6.40 (S, 1H), 12.73 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) 20.7, 44.8, 49.9, 69.4, 93.3, 98.6, 105.7, 109.0, 158.2, 162.4, 164.2, 167.2, 182.6-ppm; EI-MS (*m/z*) 248 $[M]^{+}$.

Compound 2: R_f 0.45 (MeOH:chloroform = 5:95); HPLC: R_T 2.33 min (purity; 100%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.30 (s, 3H), 2.78 (dd, *J* = 4.0, 1.6 Hz, 1H), 2.91–2.95 (m, 2H), 3.12 (s, 1H), 3.37 (s, 1H), 3.43 (s, 1H), 3.96 (dd, *J* = 10.8, 5.6 Hz, 1H), 4.12 (dd, *J* = 11.2, 4.0 Hz, 1H), 4.31–4.37 (m, 2H), 5.97 (s, 1H), 6.37 (s, 2H); ^{13}C NMR ($CDCl_3$, 100 MHz) 20.0, 44.8, 45.1, 49.9, 50.4, 69.2, 69.3, 94.4, 98.2, 109.7, 112.1, 159.8, 160.1, 162.5, 163.4, 177.3-ppm; EI-MS (*m/z*) 304 [M] + .

4.1.2. The synthesis of 7-(3-chloro-2-hydroxypropoxy)-5-hydroxy-2-methyl-4H-chromen-4-one (3)

A reaction mixture of compound **1** (0.05 g, 0.20 mmol) in aqueous 3 M-HCl in ethyl acetate was stirred at room temperature for 30 min, after which the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography (eluent: ethyl

acetate:*n*-hexane = 1:3) to give compound **3** (0.02 g, 35.1%) as a beige solid. R_f 0.47 (ethyl acetate:*n*-hexane = 1:1); mp: 169–170 °C; HPLC: R_T 4.23 min (purity; 100%); ^{1}H NMR (C*D*Cl$_3$, 400 MHz) δ 2.36 (s, 3H), 3.83–3.78 (m, 2H), 4.13–4.15 (m, 2H), 4.24–4.27 (m, 1H), 6.05 (s, 1H), 6.34 (d, J = 2.0 Hz, 1H), 6.38 (d, J = 2.0 Hz, 1H), 12.69 (s, 1H); ^{13}C NMR (C*D*Cl$_3$, 100 MHz) 20.7, 46.0, 69.1, 69.7, 93.1, 98.6, 105.8, 109.1, 158.2, 162.4, 164.0, 167.2, 182.7 ppm; EI-MS (*m/z*) 284 [M] + .

4.1.3. The synthesis of (R)-5-hydroxy-2-methyl-7-(oxiran-2-ylmethoxy)-4H-chromen-4-one (4) and 2-methyl-5,7-bis((R)-oxiran-2-ylmethoxy)-4H-chromen-4-on (5)

(*R*)-epichlorohydrin (0.73 g, 7.89 mmol) was added to a reaction mixture of 5,7-dihydroxy-2-methyl-4*H*-chromen-4-one (0.50 g, 2.63 mmol) and K_2CO_3 (0.73 g, 5.2 mmol) in DMF/acetone (9 mL/3 mL). The reaction mixture was stirred at ~80 °C for 17 h and then cooled to room temperature. After adding water, the reaction mixture was extracted with ethyl acetate. The organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and then the residue was purified by silica gel column chromatography (eluent: ethyl acetate:*n*-hexane = 1:1 → MeOH:chloroform = 5:95) to give compounds **4** (0.15 g, 23.6%) as an ivory-colored solid and **5** (0.28 g, 34.9%) as a brown syrup.

Compound 4: R_f 0.49 (ethyl acetate:*n*-hexane = 1:1); mp: 131–132 °C; HPLC: R_T 4.84 min (purity; 98.1%); ^{1}H NMR (C*D*Cl$_3$, 400 MHz) δ 2.35 (s, 3H), 2.77 (s, 1H), 2.94 (dd, J = 8.4, 4.0 Hz, 1H), 3.38 (s, 1H), 3.97 (dd, J = 11.2, 6.0 Hz, 1H), 4.30 (d, J = 11.2 Hz, 1H), 6.04 (s, 1H), 6.35 (S, 1H), 6.39 (S, 1H), 12.71 (s, 1H); ^{13}C NMR (C*D*Cl$_3$, 100 MHz) 20.7, 44.8, 49.9, 69.4, 93.3, 98.5, 105.7, 109.0, 158.2, 162.4, 164.2, 167.1, 182.7 ppm; EI-MS (*m/z*) 248 [M] + .

Compound 5: R_f 0.41 (MeOH:chloroform = 5:95); HPLC: R_T 2.98 min (purity; 98.1%); ^{1}H NMR (C*D*Cl$_3$, 400 MHz) δ 2.30 (s, 3H), 2.78 (dd, J = 4.0, 1.6 Hz, 1H), 2.91–2.95 (m, 2H), 3.13 (s, 1H), 3.38 (s, 1H), 3.43 (s, 1H), 3.96 (dd, J = 10.8, 5.6 Hz, 1H), 4.12 (dd, J = 11.6, 4.0 Hz, 1H), 4.31–4.38 (m, 2H), 5.98 (s, 1H), 6.46 (s, *2*H); ^{13}C NMR (C*D*Cl$_3$, 100 MHz) 20.0, 44.8, 45.2, 49.9, 50.4, 69.3, 72.2, 94.5, 98.3, 100.6, 112.1, 159.8, 160.0, 162.5, 163.3, 177.3 ppm; EI-MS (*m/z*) 304 [M] + .

4.1.4. The synthesis of (S)-7-(3-chloro-2-hydroxypropoxy)-5-hydroxy-2-methyl-4H-chromen-4-one (6)

A reaction mixture of **4** (0.03 g, 0.12 mmol) in aqueous 3 M-HCl in ethyl acetate (2 mL) was stirred at room temperature for 30 min, after which the solvent was removed under reduced pressure. The residue was treated with ether and the resulting solid was collected to give compound **6** (0.03 g, 87.2%) as a beige solid. R_f 0.35 (ethyl acetate:*n*-hexane = 1:1); mp: 169–170 °C; HPLC: R_T 4.34 min (purity; 97.7%); ^{1}H NMR (C*D*Cl$_3$, 400 MHz) δ 2.36 (s, 3H), 3.72–3.83 (m, 2H), 4.12–4.14 (m, 2H), 4.23–4.27 (m, 1H), 6.04 (s, 1H), 6.35 (d, J = 2.0 Hz, 1H), 6.38 (d, J = 2.0 Hz, 1H), 12.82 (s, 1H): ^{13}C NMR (C*D*Cl$_3$, 100 MHz) 20.7, 46.0, 69.1, 69.7, 93.1, 98.6, 105.9, 109.2, 158.3, 162.5, 164.0, 167.2, 182.7 ppm; EI-MS (*m/z*) 284 [M] + .

4.1.5. The synthesis of (S)-5-hydroxy-2-methyl-7-(oxiran-2-ylmethoxy)-4H-chromen-4-one (7) and 2-methyl-5,7-bis((S)-oxiran-2-ylmethoxy)-4H-chromen-4-one (8)

(*S*)-epichlorohydrin (0.73 g, 7.89 mmol) was added to a reaction mixture of 5,7-dihydroxy-2-methyl-4*H*-chromen-4-one (0.50 g, 2.63 mmol) and K_2CO_3 (0.73 g, 5.2 mmol) in DMF/acetone (9 mL/3 mL). The reaction mixture was stirred at ~80 °C for 17 h and then cooled to room temperature. After adding water, the reaction mixture was extracted with ethyl acetate. The organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and then the residue was purified by silica gel column chromatography (eluent: ethyl acetate:*n*-hexane = 1:1 → MeOH:chloroform = 2:98) to give compounds **7** (0.16 g, 24.1%) as an ivory-colored solid and **8** (0.27 g, 34.1%) as a brown syrup.

Compound 7: R_f 0.49 (ethyl acetate:*n*-hexane = 1:1); mp: 131–132 °C; HPLC: R_T 4.42 min (purity; 99.6%); ^{1}H NMR (C*D*Cl$_3$, 400 MHz) δ 2.35 (s, 3H), 2.77 (S, 1H), 2.94 (S, 1H), 3.37 (S, 1H), 3.97 (d, J = 10.8 Hz, 1H), 4.30 (d, J = 11.2 Hz, 1H), 6.03 (S, 1H), 6.34 (S, 1H), 6.39 (S, 1H), 12.71 (s, 1H); ^{13}C NMR (C*D*Cl$_3$, 100 MHz) 20.7, 44.8, 49.9, 69.4, 93.2, 98.5, 105.7, 109.0, 158.2, 162.4, 164.2, 167.2, 182.7 ppm; EI-MS (*m/z*) 248 [M] + .

Compound 8: R_f 0.45 (MeOH:chloroform = 5:95); HPLC: R_T 2.60 min (purity; 97.4%); ^{1}H NMR (C*D*Cl$_3$, 400 MHz) δ 2.31 (s, 3H), 2.79 (d, J = 2.0 Hz, 1H), 2.94–2.96 (m, 2H), 3.14 (s, 1H), 3.39 (s, 1H), 3.44 (s, 1H), 3.98 (dd, J = 10.8, 6.4 Hz, 1H), 4.13 (dd, J = 11.6, 4.0 Hz, 1H), 4.32–4.39 (m, 2H), 5.99 (s, 1H), 6.46 (s, *2*H); ^{13}C NMR (C*D*Cl$_3$, 100 MHz) 20.0, 44.8, 45.1, 50.0, 50.3, 69.3, 94.6, 98.3, 109.7, 112.1, 159.9, 160.1, 162.5, 163.3, 177.4 ppm; EI-MS (*m/z*) 304 [M] + .

4.1.6. The synthesis of (R)-7-(3-chloro-2-hydroxypropoxy)-5-hydroxy-2-methyl-4H-chromen-4-one (9)

A reaction mixture of **7** (0.03 g, 0.12 mmol) in aqueous 3 M-HCl in ethyl acetate (2 mL) was stirred at room temperature for 30 min, after which the solvent was removed under reduced pressure. The residue was treated with ether and collected to give compound **9** (0.03 g, 79.3%) as a beige solid. R_f 0.35 (ethyl acetate:*n*-hexane = 1:1); mp: 169–170 °C; HPLC: R_T 4.39 min (purity; 97.7%); ^{1}H NMR (C*D*Cl$_3$, 400 MHz) δ 2.36 (s, 3H), 3.72–3.83 (m, 2H), 4.11–4.14 (m, 2H), 4.21–4.25 (m, 1H), 6.04 (s, 1H), 6.35 (d, J = 2.0 Hz, 1H), 6.38 (d, J = 2.0 Hz, 1H), 12.82 (s, 1H): ^{13}C NMR (C*D*Cl$_3$, 100 MHz) 20.7, 46.1, 69.1, 69.7, 93.1, 98.6, 105.9, 109.2, 158.3, 162.5, 164.0, 167.2, 182.7 ppm;: EI-MS (*m/z*) 284 [M] + .

4.1.7. The synthesis of 2-methyl-7-((R)-oxiran-2-ylmethoxy)-5-((S)-oxiran-2-ylmethoxy)-4H-chromen-4-one (10)

(*S*)-epichlorohydrin (0.04 g, 0.40 mmol) was added to a reaction mixture of **4** (0.05 g, 0.20 mmol) and Cs_2CO_3 (0.10 g, 0.30 mmol) in DMF/acetone (3 mL/3 mL). The reaction mixture was stirred at 70 °C for 17 h and then cooled to room temperature. After adding water, the reaction mixture was extracted with ethyl acetate. The organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and then the residue was purified by silica gel column chromatography (eluent: MeOH:chloroform = 2:98) to give compound **10** (0.02 g, 34.5%) as a brown syrup. R_f 0.45 (MeOH:chloroform = 5:95); HPLC: R_T 2.35 min (purity; 100%); ^{1}H NMR (C*D*Cl$_3$, 400 MHz) δ 2.31 (s, 3H), 2.78 (s, 1H), 2.92–2.95 (m, 2H), 3.14 (s, 1H), 3.38 (s, 1H), 3.44 (s, 1H), 3.99 (brs, 1H), 4.14 (d, J = 11.2 Hz, 1H), 4.32–4.39 (m, 2H), 5.99 (s, 1H), 6.46 (s, *2*H); ^{13}C NMR (C*D*Cl$_3$, 100 MHz) 20.0, 29.9, 44.8, 45.1, 50.0, 50.4, 69.3, 94.6, 98.2, 112.1, 159.9, 160.1, 162.5, 163.3, 177.3 ppm; EI-MS (*m/z*) 304 [M] + .

4.1.8. The synthesis of 2-methyl-5-((R)-oxiran-2-ylmethoxy)-7-((S)-oxiran-2-ylmethoxy)-4H-chromen-4-one (11)

(*R*)-epichlorohydrin (0.04 g, 0.40 mmol) was added to a reaction mixture of **7** (0.05 g, 0.20 mmol) and Cs_2CO_3 (0.10 g, 0.30 mmol) in DMF/acetone (3 mL/3 mL). The reaction mixture was stirred at 70 °C for 21 h and then cooled to room temperature. After adding water, the reaction mixture was extracted with ethyl acetate. The organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and then the residue was purified by silica gel column chromatography (eluent: MeOH:chloroform = 2:98) to give compound **11** (0.02 g, 39.4%) as a brown syrup. R_f 0.45 (MeOH:chloroform = 5:95); HPLC: R_T 2.61 min (purity; 97.3%); ^{1}H NMR (C*D*Cl$_3$, 400 MHz) δ 2.30 (s, 3H), 2.78 (s, 1H), 2.92–2.95 (m, 2H), 3.13 (s, 1H), 3.38 (s, 1H), 3.44 (s, 1H), 3.97 (dd, J = 10.8, 6.0 Hz, 1H), 4.13 (dd, J = 11.6, 4.0 Hz, 1H), 4.31–4.39 (m, 2H), 5.98 (s, 1H), 6.45 (s, *2*H); ^{13}C NMR (C*D*Cl$_3$, 100 MHz) 20.0, 29.9, 44.8, 45.1, 50.0, 50.5, 69.3, 94.4, 98.2, 109.8, 112.1, 159.9, 160.1, 162.5, 163.4, 177.3 ppm; EI-MS (*m/z*) 304 [M] + .

4.2. The DNA topoisomerase I and IIα relaxation assays

Topoisomerases I and II (topo I and IIα) inhibition were measured by assessing the relaxation of supercoiled pBR322 plasmid DNA, as has been described previously [33]. The mixture of 100 ng of supercoiled DNA pBR322 (Thermo Fisher Scientific™, USA) and one unit of human topo I (TopoGen™, USA) or topo IIα (USB Corp., USA) was incubated in the absence or presence of the compounds prepared at 37 °C for 1 h in the reaction buffer (topo I: 10 mM Tris-HCl (pH 7.9) containing 1 mM EDTA, 0.15 M NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, and 5% glycerol; topo IIα: 10 mM Tris-HCl (pH 7.9) containing 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/mL BSA, and 1 mM adenosine triphosphate). The reaction mixture with a final volume of 10 μL was terminated by adding 2.5 μL of the stop solution (topo I: 0.5% sarkosyl containing 0.00025% bromophenol blue and 2.5% glycerol; topo IIα: 0.7 mM EDTA). The reaction mixture was then electrophoresed on a 0.8% agarose gel at 50 V for 50 min with 1X Tris-acetate-EDTA running buffer. The gels were stained for 10 min in an aqueous solution of ethidium bromide (0.5 μg/mL). The DNA bands were visualized by transillumination with UV light and quantitated using an Alpha Tech Imager™ (Alpha Innotech Corporation, USA). Camptothecin (Sigma, USA) and etoposide (Sigma, USA) were used as positive controls (topo I and topo II inhibitors, respectively).

4.3. The cell culture and antiproliferative activity assay

MCF7 (human breast adenocarcinoma), HeLa (human cervix adenocarcinoma), DU145 (human prostate cancer), HCT15 (human colorectal adenocarcinoma), and K562 (human myelogenous leukemia) were purchased from the Korean Cell Line Bank (Seoul, Korea). MCF7, HCT15, DU145, and K562 were grown in RPMI 1640 Medium (Hyclone™, USA), while HeLa (human cervical cancer cell line) was grown in DMEM medium (Hyclone™, USA) supplemented with 10% fetal bovine serum (Hyclone™, USA) and 1% penicillin-streptomycin (Thermo Fisher Scientific™, USA) [33]. All cancer cell lines were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. The medium was changed every two days. Cell viability was evaluated by using the Cyto X cell viability assay kit (LPS Solution Corporation, Korea) to measure the cell antiproliferative activity of the compounds with various cancer cell lines. MCF7, HeLa, DU145, HCT15, and K562 cells were seeded in 96-well plates at a density of 10^4 cells per well and grown for 1 day at 37 °C. After incubating the cells for 4 h in serum-free medium, they were treated with different concentrations of the compounds for 48 h. 5 μL of Cyto X was added to each well, after which they were incubated at 37 °C for an additional 2 h. The absorbance of each well was determined using an ELISA microplate reader (VERSAmax, Molecular Devices) at a wavelength of 450 nm. The IC_{50} values were evaluated using the Table curve 2D package in SPSS (SPSS Inc, Chicago).

4.4. The cell cycle analysis

The K562 cells were seeded at a density of 5×10^5 cells per 60 mm dish. After reaching 80% confluence, the cells were treated with each compound at various concentrations and for varying times [34]. They were then washed with phosphate-buffered saline (PBS; pH 7.4) and harvested by centrifugation at 2000 rpm for 15 min. The cell pellet was fixed with 70% ethanol and then incubated at −20 °C for at least 3 h. The fixed cells were centrifuged at 3000 rpm for 3 min and mixed with 500 μL of a PBS-staining solution (propidium iodide (PI) 50 mg/mL, RNase 0.1 mg/mL, and Triton X-100 0.05% in PBS) and incubated at 37 °C for 40 min. The cells were centrifuged at 3000 rpm for 3 min, and then the supernatant was removed and mixed with PBS 500 μL. Fluorescence was measured via fluorescence-activated cell sorting (FACS) on FACSCalibur flow cytometer (BD Biosciences, USA). At least 10,000 cells were measured for each sample.

4.5. The DNA interstrand cross-linking assay

The DNA cross-linking property of each compound was tested using linearized pBR322 plasmid DNA and denaturing 1.2% alkaline agarose gel electrophoresis [18]. The linear plasmid DNA was obtained by treating circular pBR322 plasmid DNA with *Eco*RI (Invitrogen, USA) and purified by ethanol precipitation. Alkaline agarose gel (1.2%) was prepared with a solution containing 50 mM NaCl and 1 mM EDTA (pH 8.0). 0.5 μg of the linearized pBR322 was incubated with the designated concentrations of the compound for 2 h at room temperature in buffered 10 mM Tris–HCl and 1 mM EDTA adjusted to pH 8.0. After the mixture of DNA and compound was loaded with agarose loading dye, the gel was soaked in an alkaline running buffer containing 50 mM NaOH and 1 mM EDTA. The gel was run in fresh alkaline running buffer and then neutralized for 1 h in neutralizing buffer solution containing 100 mM Tris and 150 mM NaCl adjusted to pH 7.6 with refreshing every 20 min. The gel was subsequently stained with ethidium bromide solution (2.5 μL of 10 mg/mL ethidium bromide in 50 mL of neutralizing buffer solution) and was then visualized with UV transillumination and photographed using ChemiImager™ Ready (Alpha Innotech Corp.).

4.6. Statistical analysis

The data are expressed as the means ± standard deviation (S.D.), with each experiment performed in triplicate. A comparison of the differences was conducted via an analysis of variance (ANOVA) using the Prism statistical software package (GraphPad Software, USA). A p-value < 0.05 was considered significant.

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