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Antitumor Activities of Biscoumarin and Dihydropyran Derivatives

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Abstract

Rising worldwide cancer incidence and resistance to current anti-cancer drugs necessitate the need for new pharmaceutical compounds and drug delivery system. Two novel series of biscoumarin (1-4) and dihydropyran (5-16) derivatives were synthesized via a one-pot multicomponent condensation reaction and evaluated for their antitumor activity *in vitro*. The X-ray crystal structure analysis of four representative compounds 2, 7, 10 and 13 confirmed the structures of these compounds. Compounds 1-4 showed the most potent antitumor activity among the total 16 derivatives. More interestingly, preliminary mechanism studies revealed that the most potent compound 4 induced apoptosis and arrested the cell cycle at the S phase in HUTU80 cells. Additionally, the increased accumulation of HUTU80 cells in the sub G1 peak further pointed to the occurence of the cell apoptosis. The selectivity index analysis demonstrated that all the biscoumarin compounds (SI=3.1-7.5) possess higher selectivity towards intestinal epithelial adenocarcinoma cell line (HuTu80) than positive control drug carboplatin (SI=1.6-1.8). The biscoumarin compounds also showed no obvious acute toxicity on mice.

Key words Biscoumarin, Dihydropyran, X-ray, Antitumor, Apoptosis, Acute toxicity

Cancer is one of the major public health problems worldwide representing the leading cause of morbidity and mortality in industrialized countries, with approximately 14 million new cases and 8.2 million cancer-related deaths in 2012, which are expected to rise in the future [1]. In China, according to the Annual Cancer Registry in 2013, more than 3 million new cases were diagnosed with cancer, which is equivalent to 6 patients diagnosed per minute, and the prevalence has been increasing [2, 3]. This casts great socioeconomic burdens. Despite the fact that chemotherapy is central to clinical management of cancer, failure in chemotherapy is not uncommon, mainly due to the dose-limiting toxicities, which is associated with the occurrence of drug resistance [4].

Natural products have been used for the treatment of various diseases and are becoming an important research area for drug discovery. These products, especially phytochemicals have been extensively studies and

have exhibited anti-carcinogenic activities by interfering with the initiation, development and progression of cancer through the modulation of various mechanisms including cellular proliferation, differentiation, apoptosis, angiogenesis, and metastasis [5-7]. This concept is gaining attention because it is a cost-effective alternative to cancer treatment.

However, the naturally occuring compounds generally tend to be less potent when used for prevention and treatment of cancer [8]. In order to get more effective antitumor agents, it is possible to make modifications on active chemical structures of title compounds. In the present study, two novel series of biscoumarin (1-4) and dihydropyran (5-16) derivatives were firstly synthesized (Fig. 1), their antitumor activities on intestinal epithelial adenocarcinoma cell line (HuTu80), mammary adenocarcinoma cell line (4T1) and pancreatic cancer cell line (PANC1) *in vitro* were then evaluated. In addition, selective toxicity of the biscoumarin compounds (1-4) was evaluated in normal human umbilical vein endothelial cell (HUVEC) and human embryonic kidney 293 cell (HEK293) using the MTT assay. The compound 4 that demonstrated the best antitumour action was chosen to be assayed by flow cytometry to determine its apoptotic behavior and mechanism. The acute toxicity of compounds 1-4 which have higher antitumour effects than others was also carried out in mice.

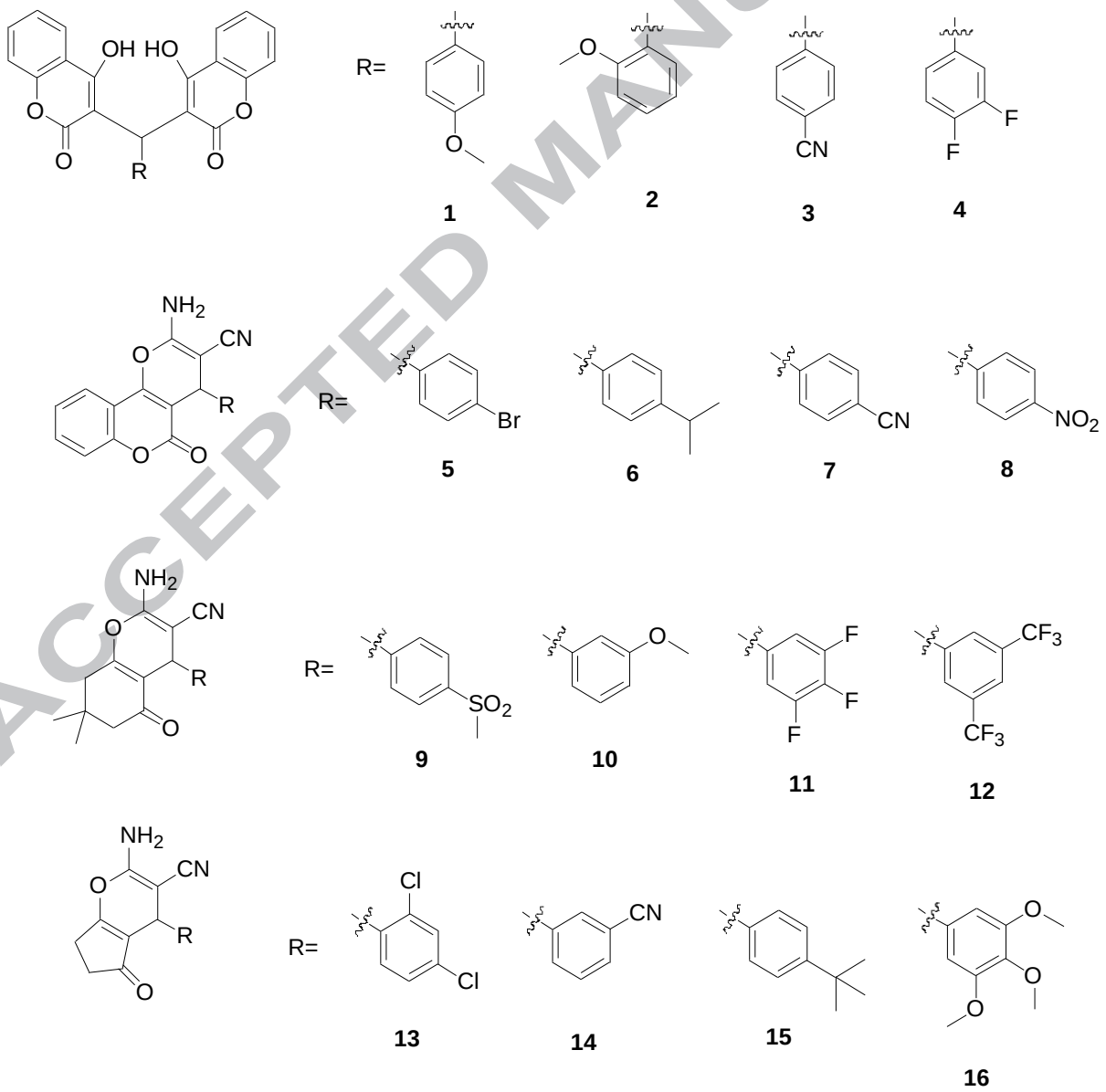
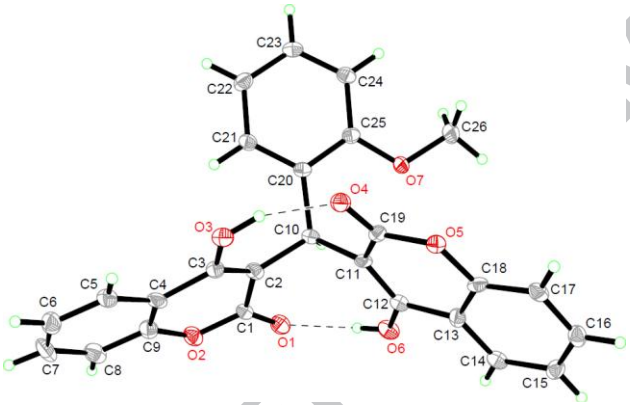


Fig. 1 Chemical structures of compounds 1-16.

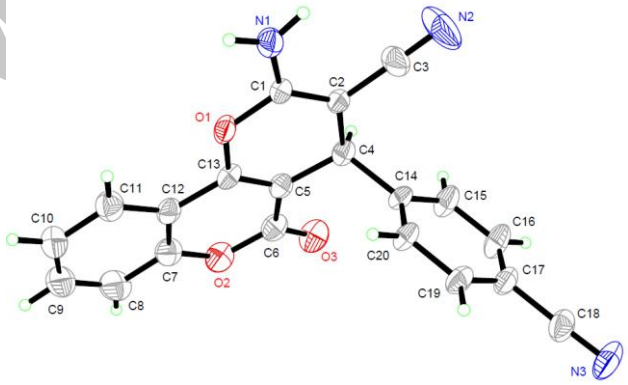
The compounds were synthesized according to general method as described in Scheme 1 and 2 (see Supplementary data). Biscoumarin 1-4 were synthesized via a one-pot two-component reaction by condensing aromatic aldehydes and 4-hydroxycoumarin in the presence of catalytic amount of piperidine in ethanol under reflux conditions. Pyran derivatives 5-16 were synthesized via a one-pot three-component reaction by condensing aromatic aldehydes, 4-hydroxycoumarin (1,1-dimethyl-3,5-cyclohexanedione or 1,3-cyclopentadione) and malononitrile in the presence of 4-(dimethylamino)pyridine (DMAP) as a highly efficient homogenous catalyst. Additionally, chemical structures of all compounds were further characterized by ^{1}H NMR and ESI-MS (see Supplementary data).

In order to further confirm the configuration of the products, single crystals of four representative compounds 2, 7, 10 and 13 were cultured for X-ray diffraction analysis. From Fig. 2 we can see that, in crystal structure of compound 2, two 4-hydroxycoumarin moieties are linked through a methylene bridge, wherein one hydrogen atom has been replaced with a 2-methoxyphenyl group; and two classical intramolecular hydrogen bonds (O_3–H_3···O_4 and O_6–H_6···O_1) between a hydroxyl group of one coumarin fragment and a lacton carbonyl group of another coumarin fragment further stabilize the whole structure.

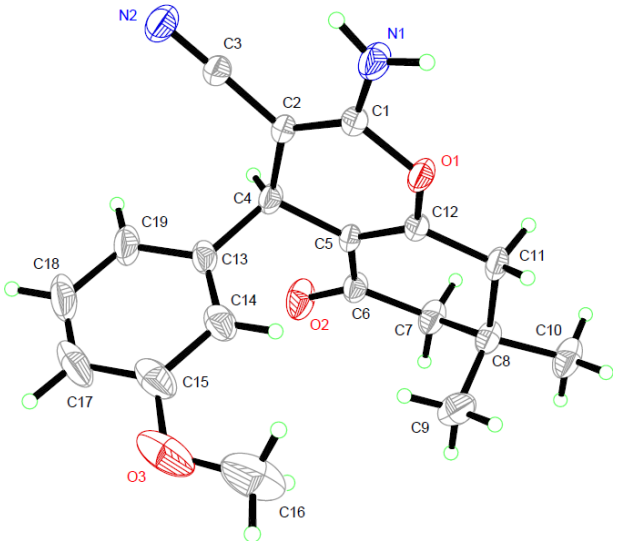
In crystal structures of compounds 7, 10 and 13, the 4*H*-pyran ring is nearly planar and the adjacent ketone ring also adopts a planar conformation. The 4*H*-pyran ring is almost perpendicular to the benzene ring and is almost coplanar with the mean plane of the ketone ring.



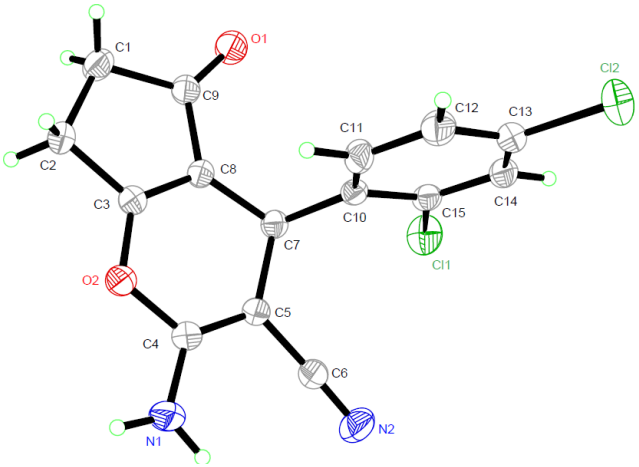
Compound 2



Compound 7



Compound 10



Compound 13

Fig. 2 Crystal structures of compounds 2, 7, 10 and 13.

Intestinal epithelial adenocarcinoma cell line (HuTu80, human origin), mammary adenocarcinoma cell line (4T1, mouse origin) and pancreatic cancer cell line (PANC1, human origin) were selected to evaluate the antitumor activities of the synthesized compounds 1-16 against different tumor types *in vitro*. Carboplatin, a standard antitumor drug, was applied to compare the potency of cytotoxicity of the tested compounds under the same experimental condition. The experimental results demonstrated that all the tested compounds had a certain degree of cell-killing activities against the three tumor cell lines and their inhibitory actions showed a evident concentration-activity relationship. Their half maximal inhibitory concentration (IC_{50}) and IC_{90} values (dose of the compound which cause a 50% and 90% reduction of survival values, respectively) are shown in Table 1. As can be seen in Table 1, according to the antitumor activity strength, the tested compounds can be divided into different groups. Among these compounds, biscoumarins 1-4 showed more potent anticancer activity against the three tested tumor cells (HuTu80, 4T1 and PANC1) with IC_{50} and IC_{90} values of 15-93.5 μg/mL and 28-201.5 μg/mL respectively; the IC_{50} and IC_{90} values of the biscoumarins 1-4 against HuTu80 are much lower than that of the positive control drug carboplatin with IC_{50} and IC_{90} values of 52.5-70.1 μg/mL and 90.7-156.2 μg/mL respectively.

Table 1 IC_{50} and IC_{90} values of compounds 1-16 and carboplatin against three tumor cell lines (μg/mL, n=3)

Drugs	HUTU80		4T1		PANC1	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Compound 1	53.8±4.1	103.2±9.8	85.7±6.4	166.6±12.7	42.5±3.7	82.5±4.9
Compound 2	17.9±1.8	109.7±8.4	52.2±±5.5	133.2±8.5	62.1±4.2	110.3±9.6
Compound 3	47.6±1.5	98.3±1.9	51.5±4.8	102±6.2	93.5±5.6	201.5±13.8
Compound 4	15±1.2	28±2.3	47.9±1.9	98.4±5.5	82.4±7.4	155±12.1
Compound 5	>300	446.6±21	>300	>500	>300	>500
Compound 6	238.1±16.4	>500	>300	>500	256.1±10.6	>500
Compound 7	>300	>500	>300	>500	>300	>500
Compound 8	97±3.5	187.2±5.6	142.2±10.6	289.4±15.8	218.4±13.4	347.8±21
Compound 9	>300	>500	>300	>500	>300	>500
Compound 10	283.3±21	>500	247.8±10.6	>500	>300	>500
Compound 11	297.3±18	>500	240.9±16.9	>500	282.8±11.5	>500
Compound 12	>300	>500	>300	>500	>300	>500
Compound 13	149.6±10.5	302.3±9.6	127.2±11.8	306.8±21.1	156.2±8.3	325.4±14.2
Compound 14	191.3±14	381.2±8.9	125.2±7.9	298.6±22	142.7±8.8	317.4±20.8
Compound 15	103±11.2	233.3±13.7	116.6±8.2	288.5±14.4	219.3±12.2	435.2±21
Compound 16	185.2±16.2	359.8±11.6	153.4±6.9	364.9±9.1	199.7±10.1	391.1±12
Carboplatin	54.3±6.9	114.7±10.2	52.5±3.8	90.7±2.6	70.1±3.5	156.2±11.4

The IC_{50} (dose of the compound which caused a 50% reduction of survival.) and IC_{90} (dose of the compound which caused a 90% reduction of survival) values were calculated from dose-response curves for each compound. IC_{50} and IC_{90} data are an average of at least 3 independent experiments. The variability of the data was less than 10%. Carboplatin was used as positive control.

One of the major hindrances for druggability of compounds with effective antitumor activity is their toxicity to normal cells. Thus, it is necessary to evaluate cytotoxicity on normal cells in the anticancer drug study. Due to the higher efficacy of anti-proliferative action against tumour cell lines, compounds 1-4 were chosen for selectivity test on normal human umbilical vein endothelial cell (HUVEC) and human embryonic kidney 293 cell (HEK293) using the MTT assay. The selectivity indexes (SI) were calculated by IC_{50} values in cancer cells divided by IC_{50} values in normal cells. The results revealed that all the tested compounds were less toxic on HUVEC or HEK293 cells in comparison with the tumor cells (Table 2). The selectivity index (SI) measures the selective cytotoxicity of a test sample against cancerous cells and the safety of sample towards normal cells. Compounds with a SI value of more than 3 are considered to have high selectivity towards the particular cancer cell line [9]. Table 3 presents the SI values of the compounds for various cancer cell lines tested. The analysis showed that all the tested compounds possess higher selectivity towards intestinal epithelial adenocarcinoma cell line (HuTu80) with SI >3. Among these compounds, compound 4 demonstrated the highest selectivity towards HuTu80 with SI>7, which was 3.5 folds better than the positive control compound carboplatin <2, followed by compound 2 with SI>6, which was 2 folds better than the positive control carboplatin.

Table 2 IC_{50} and IC_{90} values of compounds 1-4 against two normal cell lines (μg/mL, n=3)

Drugs	HUVEC		HEK293	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Compound 1	199.1±11	363.2±20.2	165.1±13.2	321.5±16.9
Compound 2	119.4±6.5	268.7±21.3	116.6±7.7	282.3±8.3
Compound 3	162.2±12.4	321.5±21.4	179.6±5.9	342.6±17.7
Compound 4	112.5±5.5	276.2±14	107.3±7.1	220.8±17.6
Carboplatin	97.4±3.2	241±11.7	89.2±4.1	222.8±15.5

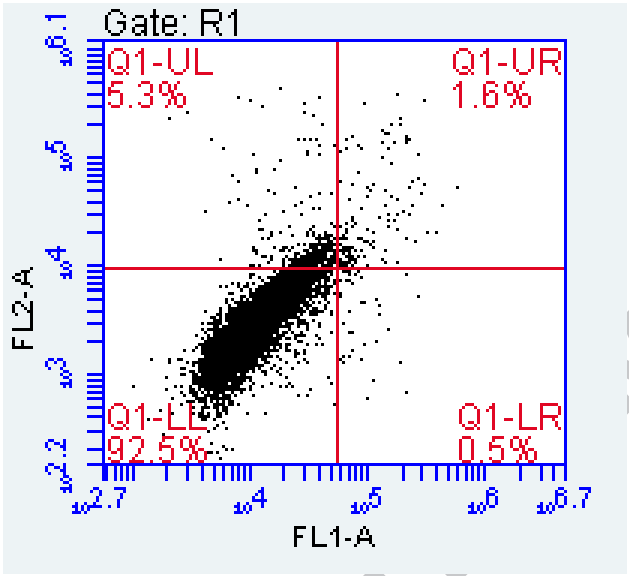
The IC_{50} (dose of the compound which caused a 50% reduction of survival.) and IC_{90} (dose of the compound which caused a 90% reduction of survival) values were calculated from dose-response curves for each compound. IC_{50} and IC_{90} data are an average of at least 3 independent experiments. The variability of the data was less than 10%. Carboplatin was used as positive control.

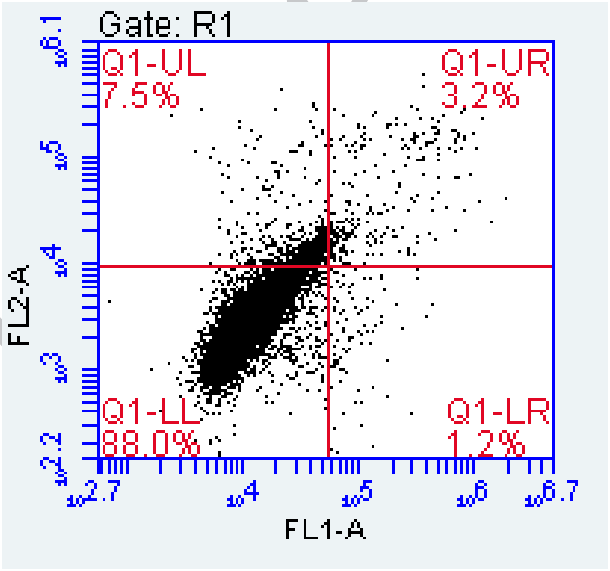
Table 3 Selectivity index for compounds 1-4 representing IC_{50} for normal cell lines/IC_{50} for cancerous cell lines

Drugs	HUVEC			HEK293		
	HUTU80	4T1	PANC1	HUTU80	4T1	PANC1
Compound 1	3.7	2.3	4.7	3.1	1.9	3.9
Compound 2	6.7	2.3	1.9	6.5	2.2	1.9
Compound 3	3.4	3.1	1.73	3.8	3.5	1.9
Compound 4	7.5	2.3	1.36	7.1	2.2	1.3
Carboplatin	1.8	1.9	1.4	1.6	1.7	1.3

Compound 4 shows the highest antiproliferative activity among all the synthesized compounds, so it was selected to assess whether cell death detected would be due to apoptosis induction. The HUTU80 cells were stained with annexin V and propidium iodide (PI) and analysized by flow cytometry. The results showed that the

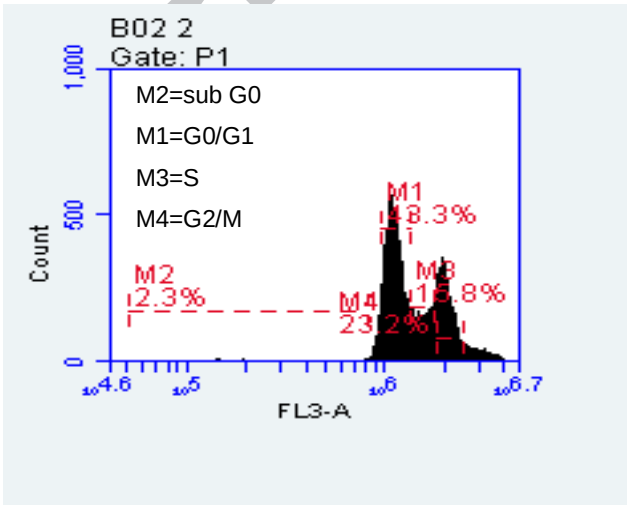
proportion of positively stained cells (regarded as apoptotic) was increased by treatment with compound 4 (Fig. 3). At 3 μg/mL of compound 4, 1.2% of HUTU80 cells transited to early apoptosis phase; 3.2% of HUTU80 cells were in late apoptosis phase and 7.5% of HUTU80 cells was in necrosis phase (Fig. 3). These results indicate that compound 4 could induce apoptosis at a lower concentration. Furthermore, the influence of the compound 4 on cell-cycle arrest of HUTU80 was also conducted by flow cytometry assay using propidium iodide (PI) staining (Fig. 4). As is seen from Fig. 4, in comparison with the control group, treatment of compound 4 (3 μg/mL) led to the obvious decrease in G1 phase (from 43.32% to 26.08%) and dramatic increase in S phase (from 15.79% to 18.48%), indicating that compound 4 is a S cell-cycle arrester. In addition, PI staining of compound 4-treated HUTU80 cells revealed increased accumulation of cells in the sub G1 peak from 2.27% to 25.06%, indicating cell apoptosis occured (Fig. 4). All these results indicate that the promising antitumor activity of compound 4 may be attributed to the S phase arrest and apoptosis.

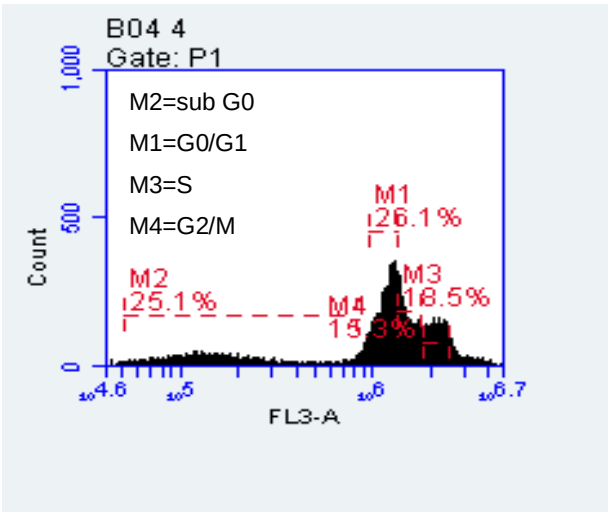




A **B**

Fig. 3 Apoptosis induction in HUTU80 cell line after 48 h treatment with compound 4 at the concentration of 3 μg/mL (B) and no treatment (vehicle control) (A).





A **B**

Fig. 4 Effect of compound 4 on apoptosis and cell cycle progression (subG1:G1:S:G2/M) in HUTU80 cells. Vehicle control (A) and drug treatment (B).

Compounds 1-4 were shown to possess higher antitumour effects among the synthesized chemical compounds. Thus these four compounds were chosen to be further studied on their acute toxicity in mice. As a result, all the animals survived the 14 day-treatment period. No physical or abnormal changes was found in the skin, fur, eyes, mucus membranes, tremors, salivation, behavior patterns, or sleep patterns. No differences were observed in kidney and liver tissue histopathology analysis between the compound-treated mice and the normal controls (Fig. 5).

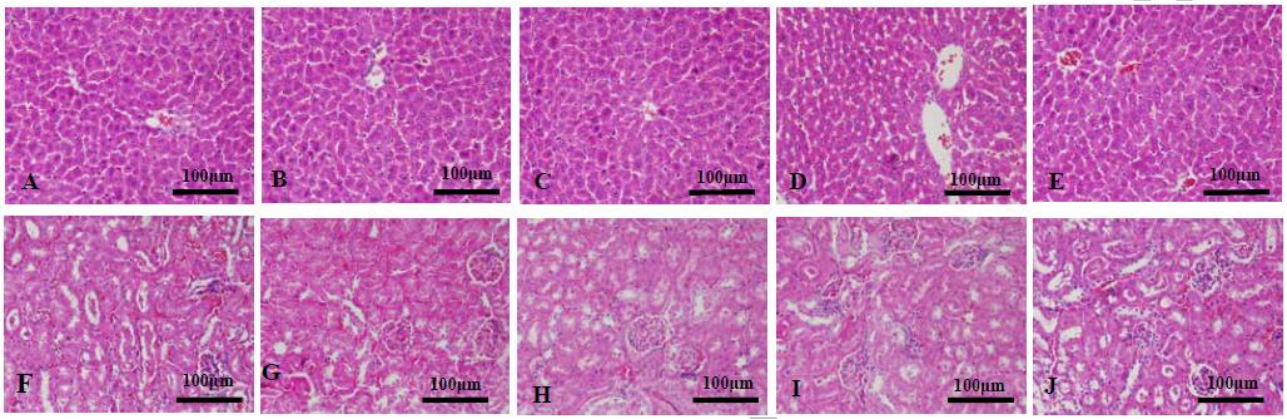


Fig. 5 Histological sections of liver (first row) and kidney (second row) in the acute toxicity test (H&E staining, 40x). Untreated mice (control group) received vehicle only (A and F). Animals treated with 500 mg/kg (B and G), (C and H), (D and I) and (E and J) are for compounds 1, 2, 3 and 4, respectively. There were no significant differences in the structures of the liver or kidneys between the treated and untreated groups.

In conclusion, two new series of biscoumarin and dihydropyran derivatives were synthesized in this work; their antitumour effects against intestinal epithelial adenocarcinoma cell line (HuTu80), mammary adenocarcinoma cell line (4T1) and pancreatic cancer cell line (PANC1) were evaluated by the MTT assay *in vitro*. On this basis, the apoptosis-inducing and cell cycle-regulating effects of the synthesized compound 4 on HuTu80 cell line were further performed. The analysis showed that all the biscoumarin compounds possess higher selectivity towards intestinal epithelial adenocarcinoma cell line (HuTu80), with compound 4 showing highest SI followed by compound 2. The biscoumarin compounds also showed no obvious acute toxicity on mice. Taken together, our results suggest that the biscoumarin compounds, such as compound 2 and 4 are potential drug candidates for cancer chemotherapy.

Of the synthesized compounds, compounds 1-4 had more potent antitumor activity against HuTu80 with the IC_{50} and IC_{90} values (15-53.8 μg/mL and 28.00-109.7 μg/mL) much lower than those of the positive control drug carboplatin (52.5-70.1 μg/mL and 90.7-156.2 μg/mL) respectively. The reason may be that two classical intramolecular O—H···O hydrogen bonds in their structures. Furthermore, the apoptosis tests using flow cytometry assay demonstrated that the best anticancer compound 4 could evoke a significant increase in the number of HUTU80 cells both in the early phase and in the late phase of cell apoptosis. Further analysis on cell cycle indicated that compound 4 is a S cell-cycle arrester of HUTU80 cells that could lead to a dramatic decrease in G1 phase and increase in S phase of the cells. Moreover, the increased accumulation of HUTU80 cells in the sub G1 peak further pointed to the occurence of the cell apoptosis.

Acknowledgments

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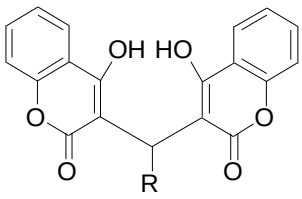

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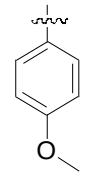

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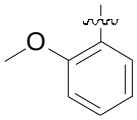

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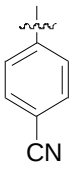

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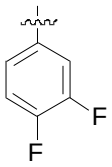

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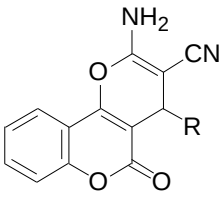

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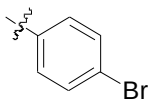

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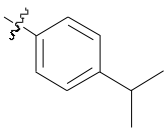

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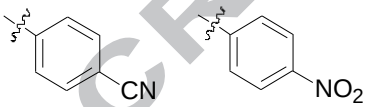
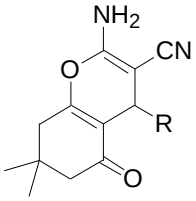


7 **8**



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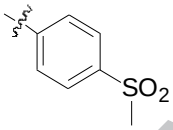

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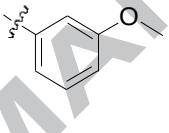

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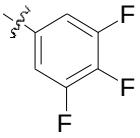

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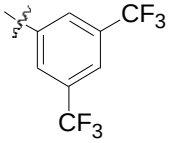

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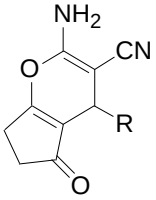

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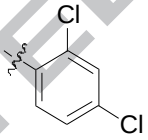

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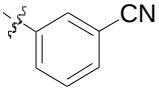

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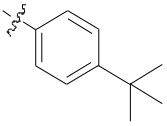

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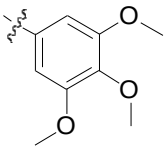

16