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Investigating the *in Vitro* Antiproliferative and Apoptosis-Inducing Effects of Pyranochromene Derivatives

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Abstract: Cancer is one of the important health problems, and researchers continue their efforts to discover new anti-cancer agents. Coumarins (chromene-2-ones), a group of natural metabolites, have shown different biological activities based on their substitutions. In this study, 15 compounds of 1,5-dihydropyrano[2,3-c]chromene were synthesized by three-component reaction and investigated for the antiproliferative activity on the breast (MCF-7), colorectal (SW48 and HT-29), lung (A549), and brain (U-87 MG) cancer cell lines as well as two normal cell lines (3T3 and HUVEC). The apoptosis/necrosis-inducing effect of the selected compounds was determined on the MCF-7 cell line by flow cytometry. The results showed that the compounds bearing a moiety on their phenyl ring's para position had potent cytotoxic effects on the tested cell lines. These compounds induced apoptosis in MCF-7 cells. The compounds were also toxic for 3T3 and HUVECs and did not display a high selectivity for tumor cells. Our results revealed that the compounds having a moiety at the para position of their phenyl ring might be suitable lead compounds for the synthesis of potent anti-cancer agents.

Keywords: anti-cancer activity; apoptosis; antitumor; pyranochromene; coumarin.

1. Introduction

Cancer is considered the most challenging problem, and the second leading cause of death worldwide [1]. Aging and population growth have increased the incidence and mortality of cancers [2]. Cancer incidence is complicated and under the effect of several factors; however, the extrinsic factors have more influence, causing increased cancer development in young adults [3]. One-third of cancer incidence and mortality is related to lung, breast, and colorectal cancers worldwide. Lung and colorectal cancers are the first and second mortal cancers for both sexes. Among females, however, breast cancer is the leading cause of cancer death [2]. From 2012 to 2016, the death rate for brain and other nervous system tumors has increased, and these types of cancers are the leading cause of cancer death among young adults [1]. Gliomas are the second most common brain tumors in adults, and glioblastomas are the most aggressive and invasive type [4, 5].

Radiotherapy and surgery are used to treat localized tumors and chemotherapy and immunotherapy for hematologic or metastatic malignancies. However, the best method of cancer treatment is still chemotherapy. Nevertheless, drug resistance is a problem in cancer

therapy, which leads to the failure of the treatment [6, 7]. Therefore, researchers continue their efforts to discover new antitumor agents. Additionally, angiogenesis, a neo-vessel growth pathway, has a role in tumorigenesis; thus, angioprevention can also be used to prevent and treat cancers, and scientists are looking for anti-angiogenesis compounds [8, 9].

Coumarins are the most diverse and abundant family of secondary metabolites exhibiting a wide range of pharmacological activities. These compounds are well known for their anticoagulant effects. Additionally, coumarins have shown antioxidant, antiviral, antiparasitic, antifungal, antibacterial, antitumor, anti-inflammatory, and anti-Alzheimer effects based on the substitution pattern [10-16]. There are extensive reports of the antitumor activity of synthetic and natural coumarin derivatives in many tumor cells, including those of colorectal, gastric, breast, and lung cancers [12]. Coumarins exert their anti-cancer effects by inhibiting angiogenesis, aromatase, telomerase, protein kinase activity, arresting cell cycle, producing oxidative stress via generating free radical species inducing apoptosis [17-20].

The present study aimed to investigate the in vitro antiproliferative and anti-apoptotic effects of 15 derivatives of synthesized 3-hydroxy coumarin.

2. Materials and Methods

2.1. Synthesis of the compounds.

A series of 1,5-dihydropyrano[2,3-c]chromene derivatives (4a-o) were synthesized by the three-component reaction of 3-hydroxycoumarin (1), malononitrile (2), and aromatic aldehydes (3a-o) in the presence of piperidine as a base in EtOH and under reflux conditions. This method was based on our previous works on environmentally friendly multi-component reactions [21-23]. The compounds were prepared as previously reported [24-29].

2.2. Cell culture.

U-87 MG (human glioblastoma), A549 (human lung), MCF-7 (human breast), SW48 and HT-29 (human colorectal) cancer cell lines, HUVEC (human umbilical vein endothelial cells), and 3T3 (mouse embryonic fibroblast) normal cell lines were purchased from the Iranian Biological Resource Center (IBRC, Tehran, Iran) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin, Biosera). The cells were incubated at 37 °C, 5% CO_2, and 95% relative humidity up to at least 80% confluent.

2.3. Cytotoxicity assay by MTT.

To evaluate the antiproliferative effects of the compounds, MTT assay was performed. The cells were trypsinized, 1×10^4 cells were cultured in each well of a 96-well microplate, and the microplates were incubated in the above-mentioned conditions for 24 h. The next day, different concentrations (from 0.1 to 500 µg/ml) of the compounds were added, and the cells were incubated for a further 24 h. Doxorubicin (the most potent anti-cancer drug) was used as the reference. At least 3 wells of the microplate were used for each concentration, and the experiment was repeated 3 times. Finally, MTT solution (5 mg/ml, Melford, England) was added to the wells, and the microplates were incubated for 3 h protected from light. Formazan crystals were solubilized in 100 µl DMSO, and the absorbance was measured at 570 nm in a

multiplate reader. The IC_{50} values were calculated using a nonlinear curve of dose-response in GraphPad® Prism version 5 from the percent of viable cells vs. logarithm of concentrations.

The selectivity index (SI) was calculated by dividing the compound's IC50 value on 3T3 by the IC_{50} value on the cancer cell line [30].

2.4. Cytotoxicity assay by trypan blue dye exclusion.

MCF-7 cells were cultured at 6-well plates (5×10^5 cells/well) and incubated for 24 h. Different concentrations of the compounds (0.1, 1, 10, 50, and 100 µg/ml) were added to each well and incubated for 24 h. The cells were trypsinized and combined with trypan blue. Then the numbers of the dead and the live cells were counted on a Hemocytometer.

2.5. Apoptosis assay by flow cytometry.

MCF-7 cells were treated with 50 µg/ml solutions of one of the compounds 4c, 4e, 4g, 4h, 4j, or 4l, or 250 µg/ml of 4n for 24 h. Detection of apoptosis in cells was carried out by flow cytometry the next day using Annexin-V-FITC/ PI (propidium iodide) Apoptosis Kit (MabTag, Germany) based on the manufacturer's protocol. Briefly, the cells were trypsinized and washed twice in ice-cold PBS. The cells were then suspended in the binding buffer to a concentration of 1×10^6 cells/ml, later stained with PE annexin-V and PI, and incubated in the dark. Cell analysis was done with the flow cytometer (CyFlow®, Sysmex Partec GmbH, Germany).

3. Results and Discussion

3.1. Chemistry.

We have synthesized a series of 1,5-dihydropyrano[2,3-c]chromene derivatives. The synthesized compounds 4a to 4o were identified by comparing their melting points and FTIR spectra with those of authentic samples. The synthesis process and the structure of the compounds are shown in Scheme 1.

3.2. Cytotoxicity assay by MTT.

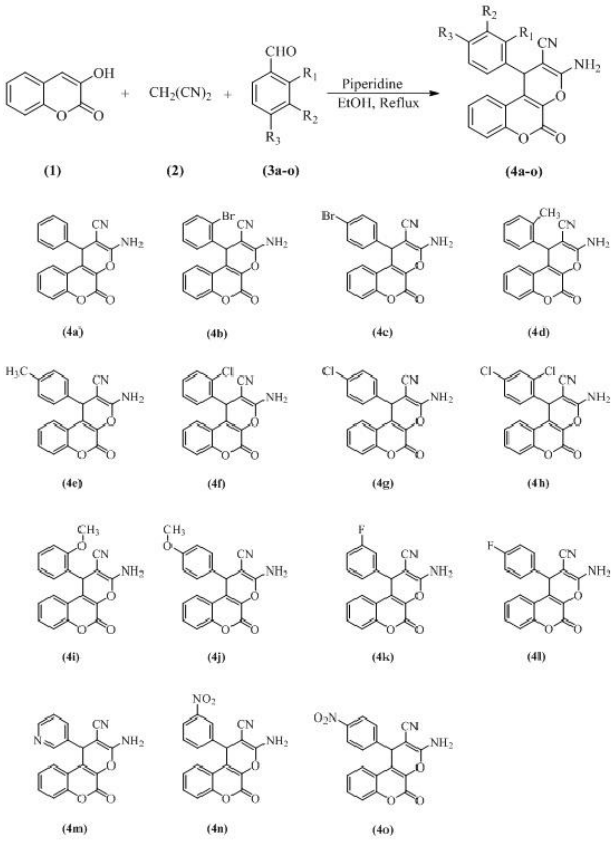
To evaluate the cytotoxic activity of the synthesized compounds, several cell lines were incubated with different concentrations of the compounds, and their viabilities were determined after 24 h. The IC_{50} values calculated from MTT assay results showed that compounds 4c, 4e, 4g, 4h, 4j, and 4l have toxic effects on some of the tested cell lines (Table 1). The compounds were significantly cytotoxic for the MCF-7 cell line. Among all the compounds, 4h showed high toxicity on all the tested cell lines, and unlike other compounds, it was toxic for the A549 cells. Most of the compounds were not toxic for the U-87 cell line except compounds 4g and 4h. It should be noted that most of these compounds were also toxic for the regular cell lines (3T3 and HUVEC). The cytotoxicity of some compounds such as 4c and 4g was more on the 3T3 than on the MCF-7 cells, and compound 4o was toxic for the 3T3 cells despite the lack of toxicity for the cancer cell lines. However, the SI (selective index) values of compounds 4e, 4j, and 4l for MCF-7 were 3.62, 5.17, and 2.48, respectively. Also, compounds 4c, 4e, 4g, 4h, 4j, and 4l were toxic for the HUVECs.

Six out of 15 compounds tested in this study were effective on growth inhibition of the cell lines. All the useful compounds had a moiety at the para position of their phenyl rings.

Compounds 4g and 4h with a Cl atom at the para position were potent antiproliferative agents. Although the additional chlorine group at the ortho site of the phenyl ring of compound 4h increased its toxicity on A549 and U-87 cells dramatically, it did not have such an effect on MCF-7 cell line. In fact, 4h was the only compound effective on A549 cell line. In addition to 4h, 4g was also effective on the U-87 cell line with a higher IC_{50}. Unlike these two chlorinated compounds, compound 4f bearing one Cl atom at an ortho position was not cytotoxic to any cell lines. It seems that the para position must be substituted in order for the ortho position to increase its cytotoxic effects. In other words, substitution at the ortho position alone is not sufficient for cytotoxicity of the compounds. This is true for other moieties (Br, CH_3, OCH_3), too, in which ortho position is not a suitable substitution.

In the same way, meta-position did not lead to the production of cytotoxic compounds as seen in compounds 4k and 4n with F and NO_2 groups at meta position, respectively. Simultaneously, their equivalents having these groups at para position (4l and 4o) were cytotoxic. It can be concluded that a moiety at the para position is necessary for toxic effects. However, the NO_2 group was not as effective as halogens, methoxy, and methyl groups. This may be related to its high electron-withdrawing effect.

Furthermore, the TPSA (topological polar surface area) of the compounds with NO_2 functional group is higher. It is seen that the higher the TPSA is, the less the in vitro toxicity will be [31]. Basanagouda *et al.* tested iodinated-4-aryloxymethyl-coumarins on breast and lung cancer cell lines and concluded that the halogenated compounds exhibited potent activity [32]. Our previous work on dihydropyrano[3,2-b]chromene derivatives, compounds having halogens, CH_3, and OCH_3 substitutions showed cytotoxic activity against cancer cell lines [33]. Similarly, there was no remarkable difference between halogens, methoxy, and methyl substitutions in this work.



Scheme 1. Synthesis of 1,5-dihydropyrano[2,3-c]chromene derivatives. (1) 3-hydroxycoumarin, (2) malononitrile, (3a-o) aromatic aldehydes, (4a-o) the synthesized compounds.

Table 1. IC_{50} (µg/ml) values of the compounds (mean ± S.E.M). Values are reported from three independent experiments.

Code	MCF-7	SW48	HT-29	A549	U87	HUVEC	3T3
4a	>500	>500	>500	>500	>500	>500	>500
4b	>500	>500	>500	>500	>500	304.25±14.15	>500
4c	21.34±2.52	47.43±5.11	70.00±7.21	293.6±19.30	399.6±26.57	13.17±0.35	11.85±0.29
4d	>500	>500	>500	>500	>500	>500	>500
4e	39.04±4.82	283.5±10.89	146.9±9.32	300.5±14.98	502±17.38	35.69±3.25	141.66±16.08
4f	>500	>500	>500	>500	>500	>500	>500
4g	24.47±1.69	44. 94±5.65	44.17±10.40	230.8±15.52	74.03±15.08	10.48±0.69	9.91±0.81
4h	15.25±1.05	19.69±4.74	21.33±6.81	55.38±8.83	18.72±5.22	16.15±1.29	17.28±2.09
4i	>500	>500	>500	>500	>500	>500	>500
4j	37.47±1.42	204.7±7.29	>500	>500	250.1±16.88	12.49±0.093	193.86±11.87
4k	>500	>500	>500	>500	>500	>500	>500
4l	38.16±3.97	93.50±6.27	87.05±8.67	206.3±8.50	305.0±15.72	29.08±0.1	94.63±7.74
4m	>500	>500	>500	>500	>500	>500	>500
4n	>500	>500	>500	>500	>500	>500	>500
4o	339.43±55.29	>500	367.6±30.37	383.5±12.89	>500	124.13±3.16	79.41±5.60
Dox	5.13 ± 0.31	5.08± 0.27	4.98 ± 0.23	6.32 ± 0.19	7.64 ± 0.34	7.36±0.54	6.25 ± 0.21

Dox: doxorubicin

3.3. Cytotoxicity assay by trypan blue dye exclusion.

The trypan blue dye exclusion test (Figure 1) confirmed the MTT assay results in that 4h lowered the percent of viable cells dramatically at the concentration of 50 µg/ml. In other words, 4h followed by 4c and 4l were the most toxic agents that disturbed the cell membrane's integrity. Surprisingly, compound 4o, which did not show cytotoxicity on the MCF-7 in the MTT assay, brought the viable cells below 50% at 100 µg/ml concentration.

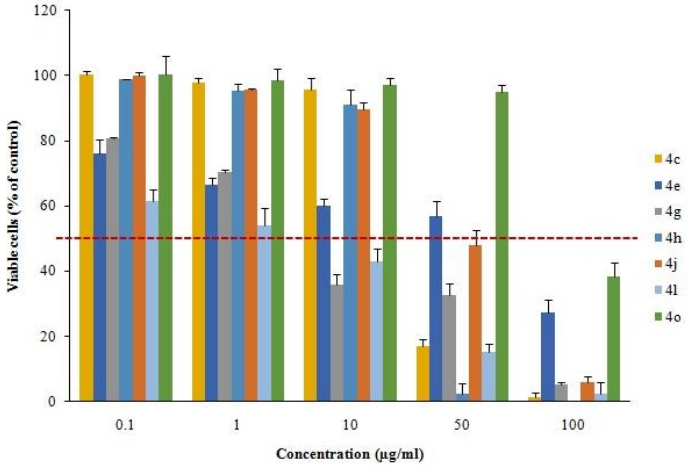


Figure 1. Cytotoxicity assay of the compounds by Trypan blue dye exclusion method. MCF-7 cells were treated with compounds for 24 h. The data represent the mean±SD.

3.4. Apoptosis assay by flow cytometry.

To investigate the compounds' apoptotic activity, MCF-7 cells treated with the compounds for 24 h were stained with a combination of Annexin V-FITC and PI and analyzed in a flow cytometer. The results of this part of the experiment are shown in Figure 2. It confirmed that all tested compounds induced apoptosis in MCF-7 cells except 4o, which induced necrosis at high concentration.

There are several reports of apoptosis-inducing activity of coumarin derivatives and hybrids. The 6-brominated coumarin hydrazide–hydrazone derivatives showed potent antiproliferative activity and induced apoptosis in a pancreatic cancer cell line [17]. O-prenylated coumarin derivatives showed antiproliferative effects on HeLa cells and induced

apoptosis after 48 h treatment; however, these compounds did not inhibit HDF normal cells [34]. Some thiazolylpyrazolyl coumarin derivatives also were toxic for the MCF-7 cell line and induced apoptosis and cell cycle arrest in these cells [35].

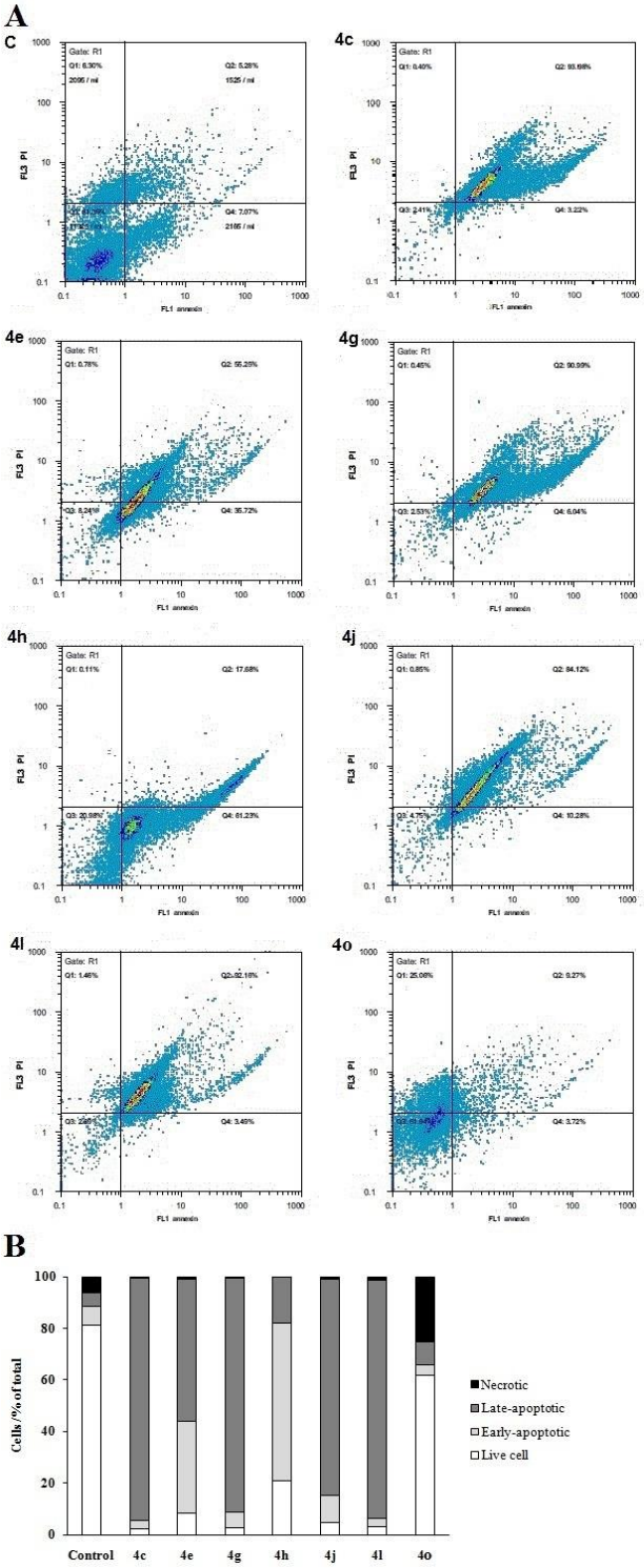


Figure 2. The apoptosis assay by flow cytometry. MCF-7 cells were treated with the compounds for 24 h. A) Dot plots showing apoptosis ratios using propidium iodide (PI) and FITC-annexin V. The Q1 quadrant represents necrotic cells (PI-positive and annexin negative), the Q2 represents late apoptotic cells (PI and annexin positive), the Q3 represents viable cells (PI and annexin negative). The Q4 represents early apoptotic cells (PI negative and annexin positive). C stands for control. B) Bar graph showing the percentage of cells in different states. Note the percentage of apoptotic cells (dark and bright grey).

4. Conclusions

Based on our results, 1,5-dihydropyrano[2,3-c]chromene derivatives can be considered suitable lead compounds for the synthesis of anti-cancer agents. A moiety on the para position of the phenyl ring showed in vitro toxic activity against cancer cell lines by introducing apoptosis. Therefore, these compounds can be considered as motivating anti-cancer agents for further study. Although HUVEC is a standard cell line, it is mostly used for evaluating the angiogenesis, preventing the potential of chemicals, so further research can be performed to investigate the anti-angiogenesis effect of these compounds.

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Conflicts of Interest

The authors declare no conflict of interest.

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