Full Paper

Synthesis and *in-vitro* Cytotoxicity of Poly-functionalized 4-(2-Arylthiazol-4-yl)-4*H*-chromenes

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A new series of 4-aryl-4*H*-chromenes bearing a 2-arylthiazol-4-yl moiety at the 4-position were prepared as potential cytotoxic agents. The *in-vitro* cytotoxic activity of the synthesized 4-aryl-4*H*-chromenes was investigated in comparison with etoposide, a well-known anticancer drug, using MTT colorimetric assay. Among them, the 2-(2-chlorophenyl)thiazol-4-yl analog **4b** showed the most potent activity against nasopharyngeal epidermoid carcinoma KB, medulloblastoma DAOY, and astrocytoma 1321N1, and compound **4d** bearing a 2-(4-chlorophenyl)thiazol-4-yl moiety at the 4-position of the chromene ring exhibited the best inhibitory activity against breast cancer cells MCF-7, lung cancer cells A549, and colon adenocarcinoma cells SW480 with IC_{50} values less than 5 μM. The ability of compound **4b** to induce apoptosis was confirmed in a nuclear morphological assay by DAPI staining in the KB and MCF-7 cells.

Keywords: Apoptosis-inducing agents / 4-Aryl-4*H*-chromenes / Cytotoxic activity / Thiazole

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Introduction

Cancer is a disease of worldwide importance and its incidence is rising. According to information from the World Health Organization (WHO), more than eleven million people are diagnosed with cancer and, also, more than 13% of overall deaths, are directly caused by cancer every year worldwide [1]. Cancer cells are characterized by unlimited replicative potential, self-sufficiency in growth signals, and insensitivity to antigrowth signals, sustained angiogenesis, metastasis, and evasion of apoptosis [2].

Apoptosis or programmed cell death is an important phenomenon for remission of damaged cells. Apoptosis can be activated by two major pathways: the extrinsic pathway, in which specific cell death receptors located on the cell surface membrane are activated by specific ligands; and the intrinsic pathway, where, primarily, mitochondria are involved [3]. Apoptosis plays a vital role in normal embryonic development as well as in adult life, such as elimination of dispensable or excess cells. It has been known that defects in the apoptosis pathways and the ability to evade cell death is one of the hallmarks of cancers, which results in uncontrollable tumor cell growth, as well as tumor resistance to chemotherapeutic agents [4]. Therefore, finding of new therapeutic agents for neoplastic diseases with focus on the apoptosis pathways is one of the top subjects in this area of research.

It has been well documented that many of the clinically useful cytotoxic agents induce apoptosis in cancer cells. The pro-apoptotic chemotherapeutic agents that target tubulin polymerization such as taxol and vinca

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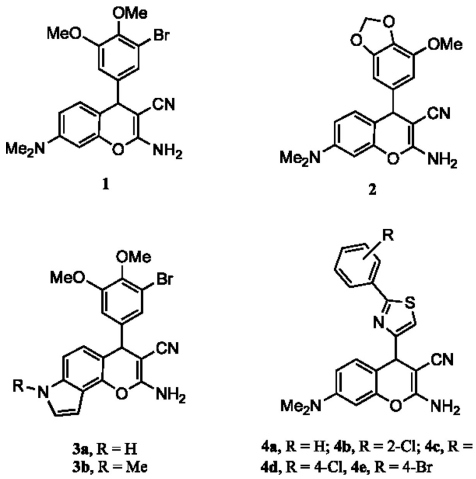


Figure 1. Structures of compounds **1**, **2**, **3a**, **b**, and **4a–e**.

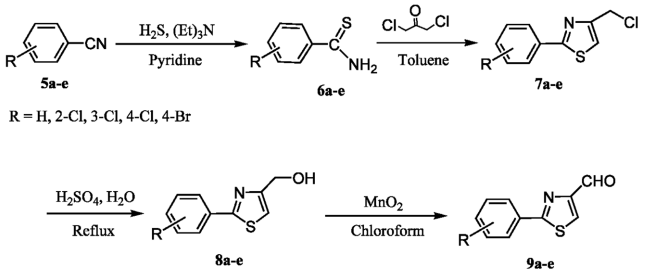
alkaloids including vincristine, vinblastine, and vinorelbine are among the most potent and commonly prescribed antineoplastic agents. The development of chemo-resistance, as well as dose-limiting neurologic and bone marrow toxicity, however, has limited the use of tubulin targeting agents. This clearly highlights the urgent need for novel chemotherapeutic agents for more effective treatment of cancer [5].

Chromene-based compounds have been reported to possess many pharmacological activities, including antibacterial properties [6, 7]; however, recent reports demonstrated the potential of 4-aryl-4*H*-chromenes **1–3** as apoptosis inducers (Fig. 1). These compounds were found to be tubulin destabilizers, binding at or close to the binding site of colchicine. They were also active in drug-resistant cancer cell lines including the paclitaxel-resistant, multi-drug resistant tumor cells, and were found to be highly active in several anticancer animal models [8, 9]. On the other hand, a diverse group of compounds having a thiazole ring have been reported as cytotoxic agents [10–13]. With these in mind, we decided to synthesize new poly-substituted 4*H*-chromenes bearing a 2-arylthiazol-4-yl moiety at the 4-posision as potential cytotoxic agents. Thus, we describe herein the synthesis of polyfunctionalized 4-(2-arylthiazol-4-yl)-4*H*-chromenes **4** (Fig. 1) and their *in-vitro* cytotoxicity against a variety of human cancer cell lines.

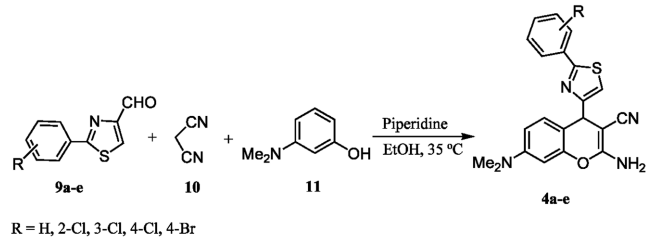
Results and discussion

Chemistry

The synthetic pathways for synthesis of key intermediates **9a–e** and target compounds 2-amino-3-cyano-7-(dimethylamino)-4-(2-arylthiazol-4-yl)-4*H*-chromenes **4a–e**



Scheme 1. Synthesis of key intermediates 2-arylthiazole-4-carboxaldehydes **9a–e**.



Scheme 2. One-pot synthesis of 4-(2-arylthiazol-4-yl)-4*H*-chromenes **4a–e**.

are outlined in Scheme 1 and Scheme 2, respectively. Benzonitrile derivatives **5a–e** were converted to thiobenzamides **6a–e**, which reacted with 1,3-dichloroactone and were converted to the corresponding chloromethylthiazole derivatives **7a–e**. The hydroxylated compounds **8a–e** were obtained by acidic hydrolysis of chloromethylthiazole derivatives **7a–e**. Oxidation of the alcohols **8a–e** by using MnO_2 afforded the desired thiazole-4-carboxaldehyde intermediates **9a–e** (Scheme 1) [14]. One-pot three-component condensation of the thiazole-4-carboxaldehydes **9a–e**, malonitrile **10** and 3-(dimethylamino) phenol **11** in the presence of piperidine in EtOH afforded target compounds **4a–e** (Scheme 2) [15, 16].

In-vitro cytotoxic and apoptosis-inducing activity

The synthesized compounds **4a–e** were tested against a panel of eight human tumor cell lines including MCF-7 (breast cancer), A549 (lung cancer), KB (nasopharyngeal epidermoid carcinoma), Hep-G2 (liver carcinoma), SW-480 (colon adenocarcinoma), U87-MG (glioblastoma), 1321N1 (astrocytoma), and DAOY (medulloblastoma). The percentage of growth inhibitory activity was evaluated using the MTT colorimetric assay in comparison with etoposide as standard drug. For each compound, the 50% inhibitory concentration (IC_{50}) was determined and is reported in Table 1. A short glance at the obtained results revealed that all compounds showed IC_{50} values less than 36.3 μM against all tested cell lines. All compounds displayed good activity against breast cancer

Table 1. Cytotoxic activity (IC_{50}, in μM) of compounds **4a–e** against different cell lines in comparison with etoposide.

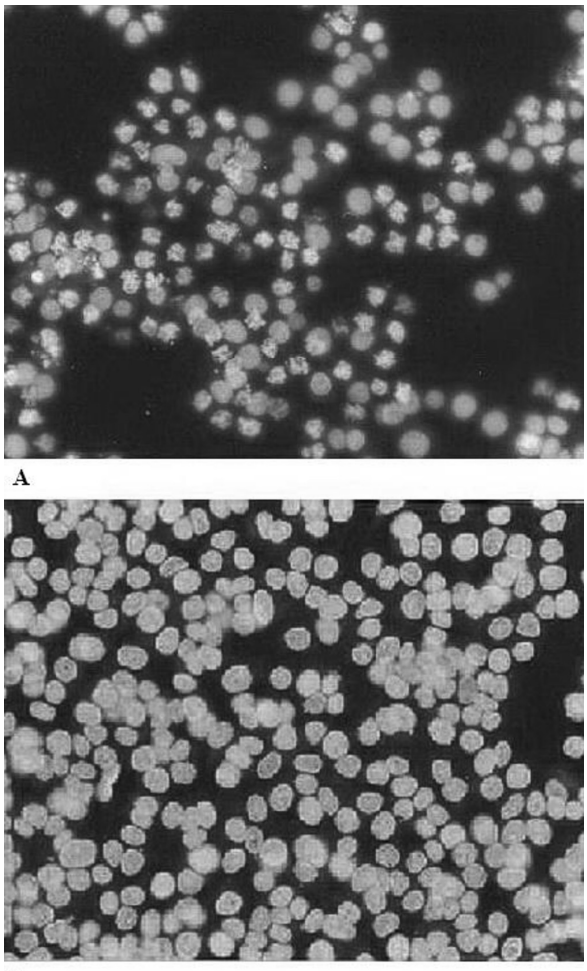
Cell line	4a	4b	4c	4d	4e	Etoposide
MCF-7	5.6 ± 1.41	2.7 ± 0.4	5.8 ± 0.58	0.36 ± 0.02	3.2 ± 0.72	0.54 ± 0.11
A549	11.4 ± 3.9	8.7 ± 2.5	10.5 ± 3.6	4.1 ± 1.7	11.8 ± 2.3	0.6 ± 0.43
SW480	28 ± 4.4	4.8 ± 1.9	17.4 ± 4.7	4.2 ± 1.1	6.6 ± 0.26	5.2 ± 0.7
Hep-G2	26.8 ± 2.7	6.4 ± 2.4	2.2 ± 0.63	3.8 ± 0.97	6.6 ± 0.88	1.1 ± 0.89
U87-MG	34.7 ± 11.4	5.9 ± 0.78	31.5 ± 9.8	6.4 ± 0.31	3.7 ± 0.29	4.4 ± 0.46
KB	28.3 ± 11.2	2.05 ± 0.17	31 ± 11.9	12.5 ± 3.1	7.3 ± 1.9	0.76 ± 0.19
DAOY	30 ± 8.1	1.8 ± 0.17	28 ± 0.7	13.9 ± 5.3	12.5 ± 1.8	11.1 ± 0.65
1321N1	36.3 ± 19.1	5.0 ± 2.9	9.3 ± 4.4	10.4 ± 2.2	9.4 ± 5.7	4.9 ± 0.54

cells MCF-7 with IC_{50} < 5.8 ± 0.58 μM. Compound **4d** was superior in inhibiting the growth of MCF-7 with an IC_{50} value of 0.36 ± 0.02 μM, being equipotent to the reference drug etoposide. Compound **4d** was also the most potent compound against lung cancer cells A549 and colon adenocarcinoma cells SW480 with IC_{50} values less than 4.2 ± 1.1 μM. The inhibitory activity of compound **4d** against SW480 was statistically comparable to that of etoposide. In the case of liver carcinoma Hep-G2, all derivatives with the exception of **4a** exhibited good inhibitory activity with IC_{50} values ranging from 0.22 to 6.6 μM. The 3-chloro- analog **4c** was the most potent compound against Hep-G2 (IC_{50} = 2.2 ± 0.63 μM). Against glioblastoma cells U87-MG, the 4-bromo- derivative **4e** exhibited the best growth inhibitory activity superior to that of the reference drug. Compound **4b** bearing a 2-chloro- substituent showed the most potent activity against nasopharyngeal epidermoid carcinoma KB, medulloblastoma DAOY, and astrocytoma 1321N1. Its activity against DAOY (IC_{50} = 1.8 ± 0.17 μM) was six-fold better than that of etoposide (IC_{50} = 11.1 ± 0.65 μM).

The comparison of IC_{50} values of the halo-substituted compounds **4b–e** and the unsubstituted compound **4a** demonstrated that the substitution with halogen in different positions of the phenyl ring generally increased the activity profile. Furthermore, the type and position of the halogen atom at the phenyl ring attached to the thiazole seemed to have a variable influence on the cytotoxic activity against various cell lines.

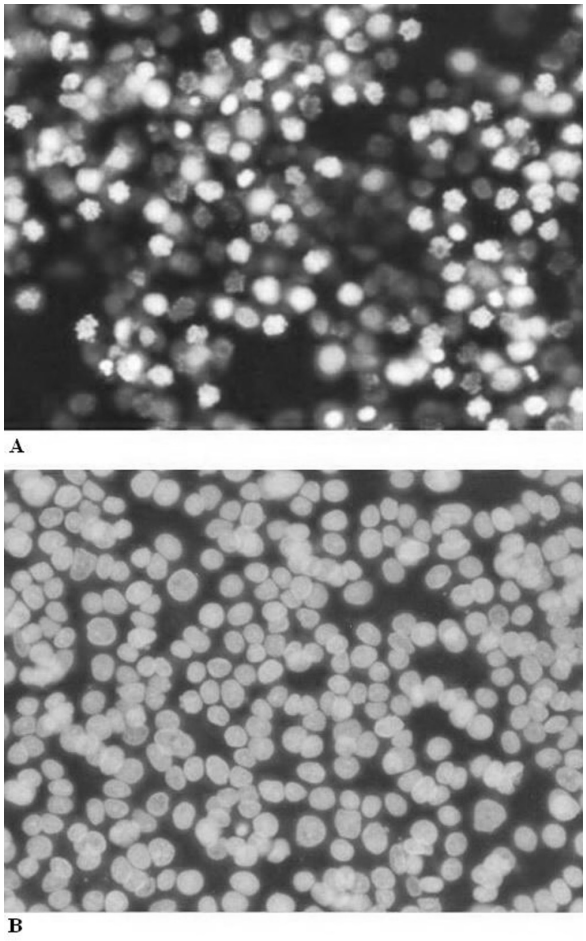
The cytotoxic activities of compounds **4a–e** against normal mouse fibroblasts (NIH/3T3) were also assessed using the MTT colorimetric assay. No cytotoxic activity was observed against this normal cell line at 10 μM concentration (IC_{50} > 10 μM). These results revealed the remarkable selectivity of active compounds against cancer cell lines.

The ability of the selected compound **4b** to induce apoptosis was confirmed in a nuclear morphological assay by DAPI staining in the KB and MCF-7 cells. The KB and MCF-7 cells were treated with 1 μg/mL or 5 μg/mL of compound **4b** for 16 h followed by staining with DAPI, a fluorescent DNA probe. The apoptotic cells are characterized by shrunken and fragmented nuclei with condensed chromatin. The results of this assay indicated that compound **4b** induced apoptosis in KB cells at 1 μg/mL (Fig.



Nasopharyngeal epidermoid carcinoma cells (KB) were treated with 1 μg/mL of compound **4b** (Fig. 2A) or 0.1% of DMSO as vehicle (Fig. 2B) for identical incubation times (16 h) and stained with DAPI. The apoptotic activity of the selected compound is confirmed by the presence of shrunken and fragmented nuclei in the compound-treated cells (Fig. 2A).

Figure 2. Fluorescent micrographs of KB cells stained with a fluorescent DNA probe, DAPI.



MCF-7 cells were treated with 5 μg/mL of compound **4b** (Fig. 3A) or 0.1% of DMSO as vehicle (Fig. 3B) for identical incubation times (16 h) and stained with DAPI. The apoptotic activity of selected compound is confirmed by the presence of shrunken and fragmented nuclei in the compound-treated cells (Fig. 3A).

Figure 3. Fluorescent micrographs of MCF-7 cells stained with a fluorescent DNA probe, DAPI.

2A). This compound also induced fragmentation of DNA in MCF-7 cells at 5 μg/mL (Fig. 3A). In contrast, the nuclei of either KB or MCF-7 treated with vehicle appeared to be normal with dispersed chromatin (Figs. 2B and 3B, respectively).

In conclusion, we synthesized a new series of 4-aryl-4*H*-chromenes bearing a 2-arylthiazol-4-yl moiety at the 4-position with potent activity against different cancer cell lines. Among them, 2-(2-chlorophenyl) thiazol-4-yl analog **4b** showed the most potent activity against nasopharyngeal epidermoid carcinoma KB, medulloblastoma DAOY, and astrocytoma 1321N1, and compound **4d** bearing a 2-(4-chlorophenyl)thiazol-4-yl moiety at the 4-position of the chromene ring exhibited the best inhibitory activity against breast cancer cells MCF-7, lung cancer cells A549, and colon adenocarcinoma cells SW480 with IC_{50} values less than 5 μM.

Experimental

Chemistry

All starting materials, reagents, and solvents were purchased from Merck AG (Germany). The purity of the synthesized compounds was confirmed by thin layer chromatography (TLC) using various solvents of different polarities. Merck silica gel 60 F_{254} plates were applied for analytical TLC. Column chromatography was performed on Merck silica gel (70–230 mesh) for purification of the intermediate and final compounds. Melting points were determined on a Kofler hot stage apparatus (Vienna, Austria) and are uncorrected. ^{1}H-NMR spectra were recorded using a Bruker 500 spectrometer (Bruker, Rheinstatten, Germany), and chemical shifts are expressed as δ (ppm) with tetramethylsilane (TMS) as internal standard. The IR spectra were obtained on a Shimadzu 470 (Shimadzu, Tokyo, Japan) spectrophotometer (potassium bromide disks). The mass spectra were run on a Finnigan TSQ-70 spectrometer (Finnigan, USA) at 70 eV. Elemental analyses were carried out on a CHN-O-rapid elemental analyzer (Heraeus GmbH, Hanau, Germany) for C, H, and N, and the results are within ± 0.4% of the theoretical values.

*General procedure for the preparation of thiobenzamide derivatives **6a–e***

Benzonitrile derivatives **5a–e** (0.51 mol) were dissolved in 200 mL of dry pyridine and 200 mL of dry triethylamine. Then, the reaction mixture was exposed to the H_2S gas for 2–4 h. The reaction was monitored by TLC. After completion of the reaction, water was added and the mixture was acidified with diluted H_2SO_4. The mixture was extracted with ethyl acetate and the organic layer was washed with diluted HCl and brine. The organic layer was dried (Na_2SO_4) and evaporated to dryness and the obtained solid was purified by column chromatography using ethyl acetate/petroleum ether (20:1) as an eluent to afford **6a–e**.

*General procedure for the preparation of 4-chloromethyl-2-phenylthiazole derivatives **7a–e***

A mixture of **6a–e** (0.1 mol) and 1,3-dichloroacetone (0.1 mol) in toluene (200 mL) was refluxed for 2 h. After completion of the reaction, toluene was evaporated under reduced pressure and water was added. The mixture was extracted three times with ethyl acetate (50 mL). The organic phase was washed (brine) and dried (Na_2SO_4). The solvent was evaporated under reduced pressure and the residue was purified by means of column chromatography using ethyl acetate/petroleum ether (1:20) as an eluent to give **7a–e**.

*General procedure for the preparation of 4-hydroxymethyl-2-phenylthiazole derivatives **8a–e***

A suspension of 4-chloromethyl-2-phenyl thiazole derivatives **7a–e** (41 mmol) and concentrated H_2SO_4 (150 mL) in water (150 mL) was refluxed for 24–48 h. The acidic reaction was neutralized by adding 10% NaOH and, after remaining at room temperature overnight, the precipitated product was recrystallized from chloroform/petroleum ether to give pure compounds **8a–e**.

*General procedure for the preparation of 2-phenylthiazole-4-carboxaldehyde derivatives **9a–e***

A mixture of 4-hydroxymethyl-2-phenylthiazole derivatives **8a–e** (22 mmol) and MnO_2 (288 mmol) in chloroform (250 mL) was stirred at room temperature for 12 h. Then, chloroform was

evaporated and diethyl ether was added. The mixture was filtered through a packed layer of diatomaceous earth and concentrated under reduced pressure. The product was crystallized from methanol/water to afford the corresponding aldehydes **9a–e**.

*General procedure for the preparation of 2-amino-3-cyano-7-(dimethylamino)-4-(2-arylthiazol-4-yl)-4H-chromenes **4a–e***

Piperidine (10 mmol) was added to a mixture of the appropriate aldehyde **9a–e** (5 mmol), malonitrile (**10**, 5 mmol), and 3-(dimethylamino)phenol (**11**, 5 mmol) in ethanol (20 mL). The reaction mixture was stirred at 35°C for 12 h. After cooling, the precipitated solid was filtered, washed with cold ethanol, and crystallized from the same solvent.

*2-Amino-3-cyano-7-(dimethylamino)-4-(2-phenylthiazol-4-yl)-4H-chromene **4a***

Yield: 74%; m.p.: 201–203°C; IR (KBr, cm^{-1}) ν_{max}: 3375, 3134, 2847, 2187, 1654, 1562, 1516, 1403, 1244, 1106, 825, 768; ^{1}H-NMR (DMSO-d_6) δ: 2.95 (s, 6H, CH_3), 5.02 (s, 2H, NH_2), 5.17 (s, 1H, H_4 chromene), 6.59 (d, 1H, J = 2.4 Hz, H_8 chromene), 6.58 (dd, 1H, J = 8.0 and 2.4 Hz, H_6 chromene), 7.01–7.55 (m, 6H, phenyl and thiazole), 7.89 (d, 1H, J = 8.8 Hz, H_5 chromene); MS (*m*/*z*,%): 374 [M^+] (40), 214 (100), 198 (14). Anal. calcd. for $C_{21}H_{18}N_4OS$: C, 67.36; H, 4.85; N, 14.96. Found: C, 67.12; H, 4.96; N, 15.15.

*2-Amino-3-cyano-4-[2-(2-chlorophenyl)thiazol-4-yl]-7-(dimethylamino)-4H-chromene **4b***

Yield: 64%; m.p.: 186–188°C; IR (KBr, cm^{-1}) ν_{max}: 3441, 3329, 3196, 2806, 2192, 1644, 1521, 1408, 1270, 1111, 1055, 825, 753, 589; ^{1}H-NMR (DMSO-d_6) δ: 2.92 (s, 6H, CH_3), 4.67 (s, 2H, NH_2), 5.04 (s, 1H, H_4 chromene), 6.30 (d, 1H, J = 2.4 Hz, H_8 chromene), 6.60 (dd, 1H, J = 8.0 and 2.4 Hz, H_6 chromene), 7.10–7.56 (m, 5H, phenyl and thiazole), 7.90 (d, 1H, J = 8.8 Hz, H_5 chromene); MS (*m*/*z*,%): 410 [M + 2] (26), 408 [M^+] (45), 344 (15), 271 (10), 214 (100), 198 (12), 174 (12). Anal. calcd. for $C_{21}H_{17}ClN_4OS$: C, 61.68; H, 4.19; N, 13.70. Found: C, 61.92; H, 4.41; N, 13.82.

*2-Amino-3-cyano-4-[2-(3-chlorophenyl)thiazol-4-yl]-7-(dimethylamino)-4H-chromene **4c***

Yield: 78%; m.p.: 200–202°C; IR (KBr, cm^{-1}) ν_{max}: 3482, 3313, 3201, 2847, 2192, 1659, 1521, 1398, 1234, 1106, 891, 814, 758, 671; ^{1}H-NMR (DMSO-d_6) δ: 2.97 (s, 6H, CH_3), 5.01 (s, 2H, NH_2), 5.16 (s, 1H, H_4 chromene), 6.10 (d, 1H, J = 2.3 Hz, H_8 chromene), 6.52 (dd, 1H, J = 8.0 and 2.3 Hz, H_6 chromene), 7.03–7.50 (m, 5H, phenyl and thiazole), 7.91 (d, 1H, J = 8.5 Hz, H_5 chromene); MS (*m*/*z*,%): 410 [M + 2] (20), 408 [M^+] (24), 344 (8), 214 (100), 198 (12), 174 (8). Anal. calcd. for $C_{21}H_{17}ClN_4OS$: C, 61.68; H, 4.19; N, 13.70. Found: C, 61.50; H, 4.12; N, 13.45.

*2-Amino-3-cyano-4-[2-(4-chlorophenyl)thiazol-4-yl]-7-(dimethylamino)-4H-chromene **4d***

Yield: 79%; m. p.: 232–235°C; IR (KBr, cm^{-1}) ν_{max}: 3353, 3303, 3144, 2858, 2796, 2192, 1669, 1516, 1398, 1239, 1111, 1004, 825, 722, 558; ^{1}H-NMR (DMSO-d_6) δ: 2.92 (s, 6H, CH_3), 4.90 (s, 2H, NH_2), 5.28 (s, 1H, H_4 chromene), 6.05 (d, 1H, J = 2.2 Hz, H_8 chromene), 6.50 (dd, 1H, J = 8.1 and 2.3 Hz, H_6 chromene), 7.05 (s, 1H, thiazole), 7.81 (d, 2H, J = 8.7 Hz, H_2 and H_6 phenyl), 7.42 (d, 2H, J = 8.7 Hz, H_3 and H_5 phenyl), 7.89 (d, 1H, J = 8.5 Hz, H_5 chromene); MS (*m*/*z*,%): 410 [M + 2] (25), 408 [M^+] (60), 344 (92), 214 (100), 198 (10), 174 (65). Anal. calcd. for $C_{21}H_{17}ClN_4OS$: C, 61.68; H, 4.19; N, 13.70. Found: C, 61.68; H, 3.99; N, 13.81.

*2-Amino-3-cyano-4-[2-(4-bromophenyl)thiazol-4-yl]-7-(dimethylamino)-4H-chromene **4e***

Yield: 81%; m.p.: 208–210°C; IR (KBr, cm^{-1}) ν_{max}: 3349, 3134, 2796, 2182, 1654, 1521, 1398, 1239, 1122, 1065, 999, 830, 697, 553; ^{1}H-NMR (DMSO-d_6) δ: 2.96 (s, 6H, CH_3), 4.88 (s, 2H, NH_2), 5.25 (s, 1H, H_4 chromene), 6.10 (d, 1H, J = 2.2 Hz, H_8 chromene), 6.53 (dd, 1H, J = 8.0 and 2.2 Hz, H_6 chromene), 7.06 (s, 1H, thiazole), 7.84 (d, 2H, J = 8.5 Hz, H_3 and H_5 phenyl), 7.43 (d, 2H, J = 8.5 Hz, H_2 and H_6 phenyl), 7.90 (d, 1H, J = 8.5 Hz, H_5 chromene); MS (*m*/*z*,%): 454 [M + 2] (20), 452 [M^+] (25), 286 (10), 255 (28), 214 (100). Anal. calcd for $C_{21}H_{17}BrN_4OS$: C, 55.64; H, 3.78; N, 12.36. Found: C, 55.93; H, 3.66; N, 12.11.

Biological activity

Cell lines and cell culture

The synthesized compounds were tested against different human cancer cell lines including MCF-7 (breast cancer), A549 (lung cancer), KB (nasopharyngeal epidermoid carcinoma), HepG2 (liver carcinoma), SW-480 (colon adenocarcinoma), U87-MG (glioblastoma), 1321N1 (astrocytoma), and DAOY (medulloblastoma). The cytotoxic activities of the target compounds were also assessed against normal mouse fibroblast (NIH/3T3) cells. The cell lines were purchased from the National Cell Bank of Iran (NCBI). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal calf serum (Biochrom, Berlin, Germany), 100 μg/mL streptomycin, and 100 U/mL penicillin, in a humidified air atmosphere at 37°C with 5% CO_2.

Cytotoxicity assay

The *in-vitro* cytotoxic activity of each synthesized chromene derivative **4a–e** was assessed in monolayer cultures using MTT colorimetric assay [17]. Briefly, each cell line in log-phase of growth was harvested by trypsinization, resuspended in complete growth medium to give a total cell count of 25×10^3 cells/mL. 100 μL of the cell suspension was seeded into the wells of 96-well plates (Nunc, Denmark). The plates were incubated overnight in a humidified air atmosphere at 37°C with 5% CO_2. Then, 50 μL of the media containing various concentrations of the compound was added per well in triplicate. The plates were incubated for further three days. The final concentration of DMSO in the highest concentration of the applied compounds was 0.1%. Etoposide was used as positive control for cytotoxicity while three wells containing tumor cells cultured in 150 μL of complete medium were used as controls for cell viability. After incubation, 30 μL of a 2.5 mg/mL solution of MTT (Sigma-Aldrich) was added to each well and the plates were incubated for another 1 h. The culture medium was then replaced with 100 μL of DMSO and the absorbance of each well was measured by using a microplate reader at 570 nm. Each set of experiments was independently performed three times. For each compound, the concentration causing 50% cell growth inhibition (IC_{50}) compared with the control was calculated from concentration-response curves by regression analysis.

Apoptosis-inducing assay

To evaluate the growth inhibitory effect of the new compounds as a result of apoptosis, the assay of nuclear morphological change was assessed by DAPI staining. In brief, KB or MCF-7 cells were seeded at a density of 2×10^5 cells per 35-mm plate and were allowed to adhere overnight. The cells were then incubated with the test compound at a selected concentration for 16 h. The selected concentrations were based on the lowest concentration of the compound inducing 50% growth inhibitory effect in the MTT assay. After incubation, the medium was discarded and the cells were fixed with 4% paraformaldehyde for 10 min, washed with PBS, and exposed to DAPI at 1 μg/mL for 5 min. The prepared cells were examined with a fluorescence microscope. Apoptosis was defined when nuclear shrinkage, chromatin condensation, or fragmented nuclei were observed. Each set of experiments was independently performed at least four times.

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The authors have declared no conflict of interest.

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