Full Paper

2-Amino-3-cyano-4-(5-arylisoxazol-3-yl)-4*H*-chromenes: Synthesis and *In Vitro* Cytotoxic Activity

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A new series of 4-aryl-4*H*-chromenes bearing a 5-arylisoxazol-3-yl moiety at the C-4 position were prepared as potential anticancer agents. The *in vitro* cytotoxic activity of the synthesized compounds was investigated against a panel of tumor cell lines including MCF-7 (breast cancer), KB (nasopharyngeal epidermoid carcinoma), Hep-G2 (liver carcinoma), MDA-MB-231 (breast cancer), and SKNMC (human neuroblastoma) using the MTT colorimetric assay. Doxorubicin, a well-known anticancer drug, was used as positive standard drug. Among the synthesized compounds, the 5-(3-methylphenyl)isoxazol-3-yl analog (**7j**) showed the most potent cytotoxic activity against all five human tumor cell lines.

Keywords: 4-(5-Arylisoxazol-3-yl)-4*H*-chromenes / Cancer / Cytotoxic activity / Synthesis

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Introduction

Cancer has been known as the leading cause of death in economically developed countries and the second leading cause of death in developing countries for several years. Increasing incidence of cancer in economically developing countries may be a result of population aging and growth as well as adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and fast food diets [1]. The disease is characterized by the uncontrolled growth of abnormal cells which are self-sufficient in growth signals, insensitive to antigrowth signals, sustained angiogenesis, metastasis, and evasion of apoptosis [2].

Cancer therapy is based on killing cancer cells selectively without harming the normal cells. A series of regulated events is involved in cell death of both malignant and non-malignant cells [3]. Several different mechanisms of cell death, including apoptosis, necrosis, mitotic catastrophe, and autophagy have been proposed. Of these, apoptosis, or programmed cell death, is one of the best understood and most studied cell death pathways. Excessive inhibition of the normal apoptosis pathway due to cellular changes results in tumor growth, metastasis, and resistance to chemotherapeutic agents [2, 4–6]. Therefore, targeting the apoptosis pathway to find new therapeutic agents for neoplastic diseases represents an opportunity to selectively kill malignant cells while reducing systemic toxicity.

Many 4-aryl-4*H*-chromenes (**A** and **B**) have been reported to be potent apoptosis inducers (Fig. 1) [7, 8]. The proposed mechanism for these series of compounds is that they were found to be tubulin destabilizers, binding at or close to the binding site of colchicine. They have also shown activity in drug-resistant cancer cell lines including the paclitaxel-resistant and multidrug resistant tumor cells [9, 10]. In addition, diverse groups of compounds bearing the isoxazole ring have shown cytotoxic effects [11]. In continuation of our efforts to design or identify new scaffolds as cytotoxic agents [8, 12, 13], herein, we decided to introduce the isoxazole ring to the 4 position of a chromene-based structure (Fig. 1). Thus, we report the synthesis and evaluation of selected 2-amino-3-cyano-7-dimethylamino-4-(5-arylisoxazol-3-yl)-4*H*-chromenes (**7a–o**) as inhibitors of cell growth.

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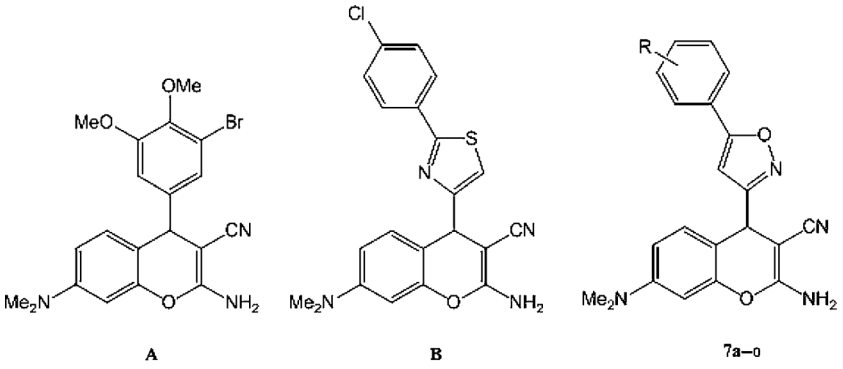


Figure 1. Potent apoptosis inducer chromene-based structures (**A** and **B**), and synthesized 4-(5-arylisoxazol-3-yl)-4*H*-chromenes (**7a–o**).

Results and discussion

Chemistry

The synthetic pathways for the synthesis of key intermediates **4a–o** and target compounds 2-amino-7-dimethylamino-4-(5-arylisoxazol-3-yl)-4*H*-chromene-3-carbonitrile **7a–o** are outlined in Scheme 1 and Scheme 2, respectively.

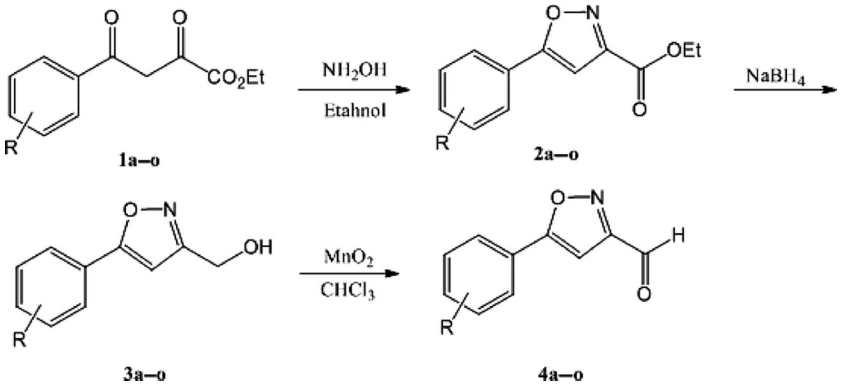
Ethyl 2,4-dioxo-4-arylbutanoate derivatives **1a–o** were reacted with hydroxylamine hydrochloride to give corresponding ethyl 5-arylisoxazole-3-carboxylates **2a–o**, which reacted with sodium borohydride and converted to 5-arylisoxazol-3-ylmethanol derivatives **3a–o**. Oxidation of the alcohols **3a–o** by using MnO_2 afforded the desired 5-arylisoxazole-3-carboxaldehydes **4a–o** (Scheme 1) [14]. One-pot three-component condensation of the 5-arylisoxazole-3-carboxaldehydes **4a–o**, malonitrile **5**, and 3-(dimethylamino)phenol **6** in the presence of piperidine in EtOH afforded target compounds **7a–o** (Scheme 2) [15, 16].

In vitro cytotoxic activity

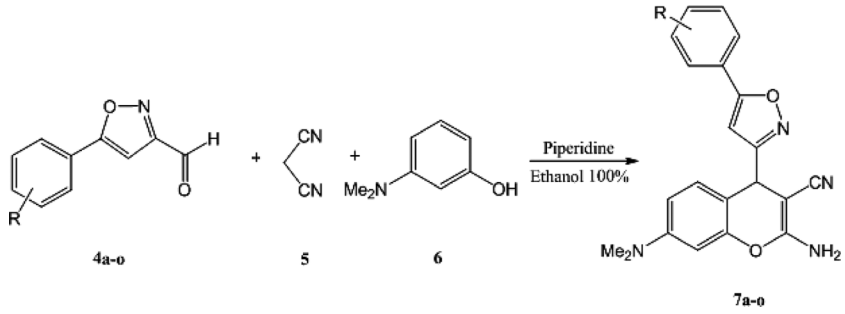
The synthesized compounds **7a–o** were tested against a panel of five human tumor cell lines including MCF-7 (breast cancer), KB (nasopharyngeal epidermoid carcinoma), Hep-G2 (liver carcinoma), MDA-MB-231 (breast cancer), and SKNMC (human neuroblastoma). The percentage of growth inhibitory activity was evaluated using the MTT colorimetric assay in comparison with doxorubicin as standard drug. For each compound, the 50% inhibitory concentration (IC_{50}) was determined and is reported in Table 1.

In general, compounds **7c** (R = 4-F), **7j** (R = 3-CH_3), and **7k** (R = 4-CH_3) displayed good activity against all tested cell lines with IC_{50} values of 6.5 ± 1.4 to 12.3 ± 0.5 μM; these compounds were also active against MDA-MB-231, SKNMC, and KB cell lines, while other compounds were inactive or moderately active. Generally, the MCF-7 and Hep-G2 cell lines were more sensitive to all the tested compounds compared to the other cell lines, in which compounds **7c**, **7j**, and **7k** exhibited higher cytotoxic activity on the mentioned cell lines.

The study of structure activity relationship of these series of 4-aryl-4*H*-chromenes revealed that 3-methylphenyl substituted analog **7j** was the most potent compound of these series against all the tested cell lines. This compound showed an IC_{50} value of 6.7 ± 2.6 μM in inhibiting the growth of MCF-7. In comparison, 4-methylphenyl substituted analog **7k** demonstrated decrease in inhibitory potency when compared to **7j** in all tested cell lines. However, in the 4-methoxyphenyl analogue (compound **7m**) changing the position of methoxy



Scheme 1. Synthesis of key intermediates 5-arylisoxazole-3-carboxaldehydes **4a–o**.

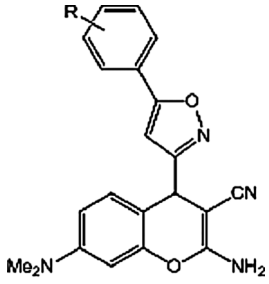


Scheme 2. One-pot synthesis of 4-(5-aryl-isoxazol-3-yl)-4*H*-chromenes **7a–o**.

group (R = 3-OCH_3) has led to loss of activity. On the other hand, 3,4-dimethoxyphenyl substitution in compound **7n** showed increased activity compared to compound **7l**. In addition, 3,4,5-tri-substituted analog **7o** was less active than **7n**, suggesting that bulky substitutions may be less preferred for cytotoxic activity in these compounds. Comparison of compound **7h** with compounds **7j** and **7l** showed that electron withdrawing groups on the 3 position can deteriorate the cytotoxic activity. Comparison of IC_{50} values of unsubstituted compound **7a** with other compounds reveals that this compound has considerable activity against all tested cell lines. In halo-substituted analogues, compound **7c** (R = 4-F) was the most potent compound, while movement of the fluorine atom to position 2 of the phenyl ring in compound **7b** led to decreased activity in all tested cell lines. Other halo- and nitro-substituted compounds showed moderate to week cytotoxic activity and the influence of the position of the halogen atom or nitro group on the cytotoxic activity was variable.

This study provides insights for further optimization of the 4-aryl-4*H*-chromene scaffold for generating novel anticancer agents.

Table 1. Cytotoxic activity (IC_{50} in μM) of compounds **7a–o** against different cancer cell lines



Compounds	R	MCF7	Hep-G2	MDA-MB-231	KB	SKNMC
7a	H	10.9 ± 0.4	11.5 ± 0.4	19.3 ± 5.4	17.5 ± 1.3	16.5 ± 1.5
7b	2-F	15.9 ± 1	13.2 ± 0.9	>30	22.3 ± 2.3	20.5 ± 4.9
7c	4-F	8.8 ± 1.1	9.5 ± 0.9	17.5 ± 12.1	11.9 ± 0.8	14.3 ± 3.8
7d	2-Cl	11.8 ± 1.6	10 ± 1.3	>30	>30	13.8 ± 1.6
7e	4-Cl	10.6 ± 4.7	15.2 ± 0.3	>30	>30	15.6 ± 2.0
7f	4-Br	12.0 ± 1.4	10.4 ± 1.6	14.7 ± 0.7	15 ± 0.1	12.0 ± 5.0
7g	2,4-Cl	13.3 ± 3.6	16.2 ± 3.8	>30	>30	>30
7h	3-NO_2	>30	26.7 ± 4.6	>30	>30	>30
7i	4-NO_2	22.6 ± 2.6	16.1 ± 4.5	>30	>30	>30
7j	3-CH_3	6.7 ± 2.6	6.5 ± 1.4	12.0 ± 4.1	11.5 ± 0.6	8.7 ± 0.9
7k	4-CH_3	9.8 ± 0.9	10.9 ± 0.1	12.1 ± 2.6	11.6 ± 4.9	12.3 ± 0.5
7l	3-OCH_3	16.1 ± 1.3	16.3 ± 3.2	>30	>30	23.6 ± 1.7
7m	4-OCH_3	>30	>30	>30	>30	>30
7n	3,4-OCH_3	15.1 ± 0.9	12.7 ± 1.0	20.1 ± 6.5	12.2 ± 0.7	12.6 ± 0.6
7o	3,4,5-OCH_3	18.5 ± 1.2	18.5 ± 7.5	>30	>30	>30
Doxorubicin	–	1.4 ± 0.4	0.5 ± 0.1	0.93 ± 0.4	4.3 ± 0.2	1.4 ± 0.3

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 F254 plates were applied for analytical TLC. 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, Rheinstetten, Germany), and chemical shifts are expressed as *d* (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 FinniganTSQ-70 spectrometer (Finnigan, USA) at 70 eV. Elemental analyses were carried out on a CHNO 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 preparation of 5-substituted phenyl isoxazole-3-carboxylate **2a–o***

A mixture of hydroxylamine hydrochloride (20 g, 0.23 mole) and the appropriate ethyl acylpyruvate (0.080 mole) in ethanol (200 mL) was heated under reflux for 3 h. The mixture was partially concentrated under vacuum, diluted with water (200 mL), and extracted with diethyl ether (3 mL × 100 mL). The organic extracts were washed with brine, then with 1 N sodium hydroxide solution (50 mL), and finally with brine. Evaporation of the solvent and distillation afforded the isoxazole esters **2a–o**.

*Ethyl 5-phenyl isoxazole-3-carboxylate **2a***

Yield: 50%; m.p.: 52–53°C; IR (KBr, cm^{-1}) υ_{max}: 3063 (C–H aromatic), 2924 (C–H, aliphatic), 1732 (C=O); ^{1}H-NMR ($CDCl_3$) δ: 7.84–7.80 (m, 2H, arom.), 7.53–7.47 (m, 3H, arom.), 6.94 (s, 1H, isoxazole), 4.48 (q, 2H, CH_2), 1.45 (t, 3H, CH_3); Anal. calcd. for $C_{12}H_{11}NO_3$: C, 66.35; H, 5.10; N, 6.45. Found: C, 66.42; H, 5.28; N, 6.12.

*Ethyl 5-(2,4-dichlorophenyl)isoxazole-3-carboxylate **2g***

Yield: 57%; m.p.: 97–98°C; IR (KBr, cm^{-1}) υ_{max}: 3103 (C–H aromatic), 2986 (C–H, aliphatic), 1726 (C=O); ^{1}H-NMR ($CDCl_3$) δ: 7.93 (d, J = 8.8 Hz, 1H, H_6phenyl), 7.56 (d, J = 1.6 Hz, 1H, H_3phenyl), 7.42 (dd, J = 8.8 and 1.6 Hz, 1H, H_5phenyl), 7.34 (s, 1H, isoxazole), 4.49 (q, 2H, CH_2), 1.45 (t, 3H, CH_3); MS (*m*/*z*, %): 285 [M^+] (5), 240 (11), 239 (17), 212 (30), 175 (64), 173 (100). Anal. calcd. for $C_{12}H_9Cl_2NO_3$: C, 50.38; H, 3.17; N, 4.90. Found: C, 50.27; H, 3.04; N, 4.72.

*General procedure for preparation of 5-substituted phenyl isoxazole-3-methanoles **3a–o***

To an ice cooled and stirred solution of the isoxazole ester (**2a–o**) (10 g, 0.048 mole) in dry ethanol (100 mL) was added portion-wise sodium borohydride (4 g, 0.11 mole). The resulting solution was stirred at room temperature for 3 h, carefully acidified with 1 N hydrochloric acid, and concentrated under vacuum. The aqueous solution was extracted with diethyl ether (3 mL × 100 mL) and concentrated under vacuum and recrystallized from dichloromethane to give compounds **3a–o**.

*(5-Phenylisoxazol-3-yl)methanol **3a***

Yield: 80%; m.p.: 91–92°C; IR (KBr, cm^{-1}) υ_{max}: 3322 (OH), 3055 (C–H aromatic), 2951 (C–H, aliphatic); ^{1}H-NMR ($CDCl_3$) δ: 7.81–7.76 (m, 2H, arom.), 7.53–7.44 (m, 3H, arom.), 6.59 (s, 1H, isoxazole), 4.83 (s, 2H, CH_2); MS (*m*/*z*, %): 175 [M^+] (100), 145 (15), 105 (20), 77 (10). Anal. calcd. for $C_{10}H_9NO_2$: C, 68.56; H, 5.18; N, 8.00. Found: C, 68.42; H, 5.28; N, 8.17.

*(5-(2,4-Dichlorophenyl)isoxazol-3-yl)methanol **3g***

Yield: 84%; m.p.: 78–79°C; IR (KBr, cm^{-1}) υ_{max}: 3220 (OH), 2923 (C–H, aliphatic), ^{1}H-NMR ($CDCl_3$) δ: 7.89 (d, J = 8.4 Hz, 1H, H_6phenyl), 7.54 (d, J = 1.6 Hz, 1H, H_3phenyl), 7.39 (dd, J = 8.4 and 1.6 Hz, 1H, H_5phenyl), 7.01 (s, 1H, isoxazole), 4.85 (s, 2H, CH_2), 1.45 (t, 3H, CH_3); MS (*m*/*z*, %): 245 [M^++2] (17), 243 [M^+] (30), 242 (53), 214 (8), 212 (12), 175 (68), 173 (100). Anal. calcd. for $C_{10}H_7Cl_2NO_2$: C, 49.21; H, 2.89; N, 5.74. Found: C, 49.27; H, 3.04; N, 5.82.

*General procedure for preparation of 5-substituted phenyl isoxazole-3-carboxaldehyde **4a–o***

A mixture of 5-substituted phenyl isoxazole-3-methanoles **3a–o** (0.02 mole) and MnO_2 (0.288 mole) in chloroform (100 mL) was stirred at room temperature for 24 h. The mixture was then filtered through a pad of celite and the filtrate was concentrated under reduced pressure. The product was crystallized from methanol/water to afford the corresponding aldehydes **4a–o**.

*5-Phenylisoxazole-3-carbaldehyde **4a***

Yield: 64%; m.p.: 59–61°C; IR (KBr, cm^{-1}) υ_{max}: 3056 (C–H aromatic), 1714 (C=O); ^{1}H-NMR ($CDCl_3$) δ: 10.21 (s, 1H, C–H aldehyde), 7.85–7.80 (m, 2H, arom.), 7.53–7.48 (m, 3H, arom.), 6.91 (s, 1H, isoxazole); MS (*m*/*z*, %): 173 [M^+] (100), 171 (100), 106 (15), 77 (90), 63 (23). Anal. calcd. for $C_{10}H_7NO_2$: C, 69.36; H, 4.07; N, 8.09. Found: C, 69.25; H, 4.16; N, 8.22.

*5-(2,4-Dichlorophenyl)isoxazole-3-carbaldehyde **4g***

Yield: 52%; m.p.: 109–110°C; IR (KBr, cm^{-1}) υ_{max}: 3101 (C–H aromatic), 1716 (C=O); ^{1}H-NMR ($CDCl_3$) δ: 10.22 (s, 1H, C–H aldehyde), 7.94 (d, J = 8.8 Hz, 1H, H_6phenyl), 7.57 (d, J = 2.0 Hz, 1H, H_3phenyl), 7.42 (dd, J = 8.8 and 2.0 Hz, 1H, H_5phenyl), 7.32 (s, 1H, isoxazole); MS (*m*/*z*, %): 241 [M^+] (23), 240 (44), 175 (63), 173 (100), 144 (24), 109 (18), 68 (8). Anal. calcd. for $C_{10}H_5Cl_2NO_2$: C, 49.62; H, 2.08; N, 5.79. Found: C, 49.55; H, 2.18; N, 5.82.

*General procedure for preparation of 2-amino-3-cyano-7-dimethylamino-4-(5-arylisoxazol-3-yl)-4H-chromenes **7a–o***

Piperidine (10 mmol) was added to a mixture of the appropriate aldehyde **4a–o** (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 ethanol.

*2-Amino-3-cyano-7-dimethylamino-4-(5-phenylisoxazol-3-yl)-4H-chromene **7a***

Yield: 30%; m.p.: 214–216°C; IR (KBr, cm^{-1}) υ_{max}: 3428 and 3315 (NH_2), 2921 (C–H, aliphatic), 2197 (CN); ^{1}H-NMR ($CDCl_3$) δ: 7.73–

7.70 (m, 2H, arom.), 7.43–7.39 (m, 3H, arom.), 7.03 (d, $J = 8.4$ Hz, 1H, H_5chromene), 6.48 (dd, $J = 2.4$ and 8.4 Hz, 1H, H_6 chromene), 6.32 (s, 1H, isoxazole), 6.29 (d, $J = 2.4$ Hz, 1H, H_8chromene), 4.98 (s, 1H, H_4chromene), 4.71 (s, 2H, NH_2), 2.94 (s, 6H, 2 × CH_3); MS (*m*/*z*, %): 359 [M^++1] (5), 358 [M^+] (27), 214 (100), 198 (17), 105 (28), 77 (38), 51 (18). Anal. calcd. for $C_{21}H_{18}N_4O_2$: C, 70.38; H, 5.06; N, 15.63. Found: C, 70.71; H, 5.38; N, 15.95.

2-Amino-3-cyano-7-dimethylamino-4-(5-(2-fluorophenyl)-isoxazol-3-yl)-4H-chromene ***7b***

Yield: 40%; m.p.: 212–214°C; IR (KBr, cm^{-1}) υ_{max}: 3430 and 3315 (NH_2), 2921 (C–H, aliphatic), 2197 (CN); ^{1}H-NMR ($CDCl_3$) δ: 7.96–7.90 (m, 1H, arom.), 7.42–7.35 (m, 1H, arom.), 7.27–7.20 (m, 1H, arom.), 7.21–7.11 (m, 1H, arom.), 7.01 (d, $J = 8.4$ Hz, 1H, H_5chromene), 6.50 (d, $J = 2.4$ Hz, H_8chromene), 6.47 (dd, $J = 8.4$ and 2.4 Hz, 1H, H_6chromene), 6.30 (d, 1H, isoxazole), 5.01 (s, 1H, H_4chromene), 4.72 (s, 2H, NH_2), 2.94 (s, 6H, 2 × CH_3); MS (*m*/*z*, %): 376 [M^+] (10), 336 (4), 213 (87), 186 (14), 123 (100), 95 (34), 75 (16). Anal. calcd. for $C_{21}H_{17}FN_4O_2$: C, 67.01; H, 4.55; N, 14.89. Found: C, 67.39; H, 4.26; N, 15.11.

2-Amino-3-cyano-7-dimethylamino-4-(5-(4-fluorophenyl)-isoxazol-3-yl)-4H-chromene ***7c***

Yield: 45%; m.p.: 200–202°C; IR (KBr, cm^{-1}) υ_{max}: 3460 and 3288 (NH_2), 2920 (C–H, aliphatic), 2179 (CN); ^{1}H-NMR ($CDCl_3$) δ: 7.73–7.67 (m, 2H, arom.), 7.11 (t, 2H, arom.), 7.02 (d, $J = 8.0$ Hz, 1H, H_5chromene), 6.48 (dd, $J = 2.8$ and 8.0 Hz, 1H, H_6chromene), 6.29 (d, $J = 2.8$ Hz, 1H, H_8chromene), 6.27 (s, 1H, isoxazole), 4.98 (s, 1H, H_4chromene), 4.71 (s, 2H, NH_2), 2.94 (s, 6H, 2 × CH_3); MS (*m*/*z*, %): 376 [M^+] (5), 318 (17), 213 (90), 186 (22), 136 (35), 123 (100), 95 (68), 75 (30). Anal. calcd. for $C_{21}H_{17}FN_4O_2$: C, 67.01; H, 4.55; N, 14.89. Found: C, 66.88; H, 4.23; N, 14.89.

2-Amino-3-cyano-7-dimethylamino-4-(5-(2-chlorophenyl)-isoxazol-3-yl)-4H-chromene ***7d***

Yield: 35%; m.p.: 202–204°C; IR (KBr, cm^{-1}) υ_{max}: 3424 and 3316 (NH_2), 2921 (C–H, aliphatic), 2198 (CN); ^{1}H-NMR ($CDCl_3$) δ: 7.97–7.91 (m, 1H, arom.), 7.49–7.42 (m, 1H, arom.), 7.41–7.31 (m, 2H, arom.), 7.02 (d, $J = 8.8$ Hz, 1H, H_5chromene), 6.74 (s, 1H, isoxazole), 6.49 (dd, $J = 2.4$ and 8.8 Hz, 1H, H_6chromene), 6.29 (d, $J = 2.4$ Hz, 1H, H_8chromene), 5.01 (s, 1H, H_4chromene), 4.65 (s, 2H, NH_2), 2.94 (s, 6H, 2 × CH_3); MS (*m*/*z*, %): 394 [M^++2] (5), 392 [M^+] (15), 362 (100), 214 (76), 198 (16), 139 (18), 57 (35). Anal. calcd. for $C_{21}H_{17}ClN_4O_2$: C, 64.21; H, 4.36; N, 14.26. Found: C, 64.21; H, 4.67; N, 14.12.

2-Amino-3-cyano-7-dimethylamino-4-(5-(4-chlorophenyl)-isoxazol-3-yl)-4H-chromene ***7e***

Yield: 37%; m.p.: 199–201°C; IR (KBr, cm^{-1}) υ_{max}: 3456 and 3285 (NH_2), 2921 (C–H, aliphatic), 2181 (CN); ^{1}H-NMR ($CDCl_3$) δ: 7.65 (d, $J = 8.0$ Hz, 2H, arom.), 7.39 (d, $J = 8.0$ Hz, 2H, arom.), 7.02 (d, $J = 8.8$ Hz, 1H, H_5chromene), 6.48 (dd, $J = 2.8$ and 8.8 Hz, 1H, H_6chromene), 6.31 (s, 1H, isoxazole), 6.29 (d, $J = 2.8$ Hz, 1H, H_8chromene), 5.01 (s, 1H, H_4chromene), 4.65 (s, 2H, NH_2), 2.94 (s, 6H, 2 × CH_3); MS (*m*/*z*, %): 394 [M^++2] (8), 392 [M^+] (23), 362 (53), 214 (100), 198 (19), 139 (52), 111 (34). Anal. calcd. for $C_{21}H_{17}ClN_4O_2$: C, 64.21; H, 4.36; N, 14.26. Found: C, 64.04; H, 4.09; N, 14.52.

2-Amino-3-cyano-7-dimethylamino-4-(5-(4-bromophenyl)-isoxazol-3-yl)-4H-chromene ***7f***

Yield: 40%; m.p.: 202–204°C; IR (KBr, cm^{-1}) υ_{max}: 3457 and 3287 (NH_2), 2920 (C–H, aliphatic), 2179 (CN); ^{1}H-NMR ($CDCl_3$) δ: 7.61–7.50 (m, 4H, arom.), 7.02 (d, $J = 9.6$ Hz, 1H, H_5chromene), 6.48 (dd, $J = 4.1$ and 9.6 Hz, 1H, H_6chromene), 6.32 (s, 1H, isoxazole), 6.29 (d, $J = 4.1$ Hz, 1H, H_8chromene), 4.98 (s, 1H, H_4chromene), 4.71 (s, 2H, NH_2), 2.94 (s, 6H, 2 × CH_3); MS (*m*/*z*, %): 438 [M^++2] (47), 436 [M^+] (47), 356 (9), 281 (10), 214 (100), 198 (72), 185 (65), 155 (34). Anal. calcd. for $C_{21}H_{17}BrN_4O_2$: C, 57.68; H, 3.92; N, 12.81. Found: C, 57.95; H, 4.08; N, 12.95.

2-Amino-3-cyano-7-dimethylamino-4-(5-(2,4-dichlorophenyl)isoxazol-3-yl)-4H-chromene ***7g***

Yield: 43%; m.p.: 188–190°C; IR (KBr, cm^{-1}) υ_{max}: 3420 and 3314 (NH_2), 3109 (C–H aromatic), 2898 (C–H, aliphatic), 2198 (CN); ^{1}H-NMR ($CDCl_3$) δ: 77.89 (d, $J = 8.8$ Hz, 1H, arom.), 7.49 (d, $J = 2.0$ Hz, 1H, arom.), 7.36 (dd, $J = 2.0$ and 8.8 Hz, 1H, arom.), 7.01 (d, $J = 8.8$ Hz, 1H, H_5chromene), 6.74 (s, 1H, isoxazole), 6.48 (dd, $J = 2.4$ and 8.8 Hz, 1H, H_6chromene), 6.29 (d, $J = 2.4$ Hz, 1H, H_8chromene), 5.01 (s, 1H, H_4chromene), 4.71 (s, 2H, NH_2), 2.94 (s, 6H, 2 × CH_3); Anal. calcd. for $C_{21}H_{16}Cl_2N_4O_2$: C, 59.03; H, 3.77; N, 13.11. Found: C, 59.37; H, 4.02; N, 13.45.

2-Amino-3-cyano-7-dimethylamino-4-(5-(3-nitrophenyl)-isoxazol-3-yl)-4H-chromene ***7h***

Yield: 38%; m.p.: 178–180°C; IR (KBr, cm^{-1}) υ_{max}: 3466 and 3345 (NH_2), 2922 (C–H, aliphatic), 2188 (CN), 1527 and 1348 (NO_2); ^{1}H-NMR ($CDCl_3$) δ: 8.54–8.51 (m, 1H, arom.), 8.28–8.24 (m, 1H, arom.), 8.07 (d, 1H, arom.), 7.64 (t, 1H, arom.), 7.01 (d, $J = 8.8$ Hz, 1H, H_5chromene), 6.49 (dd, $J = 2.0$ and 8.8 Hz, 1H, H_6chromene), 6.48 (s, 1H, isoxazole), 6.29 (d, $J = 2.0$ Hz, 1H, H_8chromene), 5.02 (s, 1H, H_4chromene), 4.78 (s, 2H, NH_2), 2.96 (s, 6H, 2 × CH_3). Anal. calcd. for $C_{21}H_{17}N_5O_4$: C, 62.53; H, 4.25; N, 17.36. Found: C, 62.88; H, 4.39; N, 17.12.

2-Amino-3-cyano-7-dimethylamino-4-(5-(4-nitrophenyl)-isoxazol-3-yl)-4H-chromene ***7i***

Yield: 45%; m.p.: 208–210°C; IR (KBr, cm^{-1}) υ_{max}: 3453 and 3287 (NH_2), 2921 (C–H, aliphatic), 2179 (CN), 1519 and 1336 (NO_2); ^{1}H-NMR ($CDCl_3$) δ: 8.30 (d, $J = 8.8$ Hz, 2H, arom.), 7.91 (d, $J = 8.0$ Hz, 2H, arom.), 6.98 (d, $J = 8.0$ Hz, 1H, H_5chromene), 6.54 (s, 1H, isoxazole), 6.48 (dd, 1–H, H_6chromene), 6.30 (bs, 1H, H_8chromene), 5.65 (s, 1H, H_4chromene), 4.95 (s, 2H, NH_2), 2.94 (s, 6H, 2 × CH_3); MS (*m*/*z*, %): 403 [M^+] (26), 264 (6), 214 (100), 198 (17), 150 (47), 104 (18), 76 (20), 57 (17). Anal. calcd. for $C_{21}H_{17}N_5O_4$: C, 62.53; H, 4.25; N, 17.36. Found: C, 62.35; H, 4.11; N, 17.09.

2-Amino-3-cyano-7-dimethylamino-4-(5-(3-methylphenyl)-isoxazol-3-yl)-4H-chromene ***7j***

Yield: 25%; m.p.: 181–183°C; IR (KBr, cm^{-1}) υ_{max}: 3432 and 3320 (NH_2), 2920 (C–H, aliphatic), 2196 (CN); ^{1}H-NMR ($CDCl_3$) δ: 7.58–7.48 (m, 2H, arom.), 7.35–7.27 (m, 2H, arom.), 7.23–7.19 (m, 1H, H_5chromene), 7.03 (d, 1H, H_6chromene), 6.52–6.45 (m,1H, H_8chromene), 6.30 (s, 1H, isoxazole), 6.28 (s, 1H, H_8chromene), 4.97 (s, 1H, H_4chromene), 4.72 (s, 2H, NH_2), 2.94 (s, 6H, 2 × CH_3), 2.37 (s, 3H, CH_3); MS (*m*/*z*, %): 372 [M^+] (29), 362 (20), 214 (100), 198 (17), 71 (57), 57 (93). Anal. calcd. for $C_{22}H_{20}N_4O_2$: C, 70.95; H, 5.41; N, 15.04. Found: C, 71.22; H, 5.65; N, 14.87.

2-Amino-3-cyano-7-dimethylamino-4-(5-(4-methylphenyl)-isoxazol-3-yl)-4H-chromene ***7k***

Yield: 27%; m.p.: 182–184°C; IR (KBr, cm^{-1}) υ_{max}: 3461 and 3285 (NH_2), 2922 (C–H, aliphatic), 2179 (CN); ^{1}H-NMR ($CDCl_3$) δ: 7.60 (d, J = 8.0 Hz, 2H, arom.), 7.21 (d, J = 8.0 Hz, 2H, arom.), 7.03 (d, J = 8.8 Hz, 1H, H_5chromene), 6.48 (dd, 1H, H_6chromene), 6.29 (d, 1H, H_8chromene), 6.26 (s, 1H, isoxazole), 4.97 (s, 1H, H_4chromene), 4.74 (s, 2H, NH_2), 2.94 (s, 6H, 2 × CH_3), 2.37 (s, 3H, CH_3); MS (*m*/*z*, %): 372 [M^+] (31), 343 (1), 280 (2), 214 (100), 198 (17), 170 (5), 119 (16), 91 (16). Anal. calcd. for $C_{22}H_{20}N_4O_2$: C, 70.95; H, 5.41; N, 15.04. Found: C, 70.76; H, 5.22; N, 15.36.

2-Amino-3-cyano-7-dimethylamino-4-(5-(3-methoxyphenyl)isoxazol-3-yl)-4H-chromene ***7l***

Yield: 30%; m.p.: 216–218°C; IR (KBr, cm^{-1}) υ_{max}: 3452 and 3277 (NH_2), 2923 (C–H, aliphatic), 2177 (CN); ^{1}H-NMR ($CDCl_3$) δ: 7.37–7.23 (m, 3H, arom.), 7.03 (d, J = 8.8 Hz, 1H, H_5chromene), 6.96–6.91 (m, 1H, arom.), 6.48 (dd, J = 2.8 and 8.8 Hz, H_6chromene), 6.31 (s, 1H, isoxazole), 6.29 (d, J = 2.8 Hz, 1H, H_8chromene), 5.01 (s, 1H, H_4chromene), 4.73 (s, 2H, NH_2), 3.84 (s, 3H, OCH_3), 2.94 (s, 6H, 2 × CH_3); Anal. calcd. for $C_{22}H_{20}N_4O_3$: C, 68.03; H, 5.19; N, 14.42. Found: C, 68.37; H, 4.89; N, 14.13.

2-Amino-3-cyano-7-dimethylamino-4-(5-(4-methoxyphenyl)isoxazol-3-yl)-4H-chromene ***7m***

Yield: 30%; m.p.: 206–208°C; IR (KBr, cm^{-1}) υ_{max}: 3464 and 3288 (NH_2), 2927 (C–H, aliphatic), 2178 (CN); ^{1}H-NMR ($CDCl_3$) δ: 7.65 (dd, J = 1.6 and 6.8 Hz, 2H, arom.), 7.03 (d, J = 8.8 Hz, 1H, H_5chromene), 6.92 (dd, J = 1.6 and 6.8 Hz, 2H, arom.), 6.47 (dd, J = 2.4 and 8.4 Hz, H_6chromene), 6.29 (d, J = 2.4 Hz, 1H, H_8chromene), 6.19 (s, 1H, isoxazole), 4.95 (s, 1H, H_4chromene), 4.70 (s, 2H, NH_2), 3.84 (s, 3H, OCH_3), 2.94 (s, 6H, 2 × CH_3); Anal. calcd. for $C_{22}H_{20}N_4O_3$: C, 68.03; H, 5.19; N, 14.42. Found: C, 67.88; H, 5.47; N, 14.69.

2-Amino-3-cyano-7-dimethylamino-4-(5-(3,4-dimethoxyphenyl)isoxazol-3-yl)-4H-chromene ***7n***

Yield: 28%; m.p.: 192–194°C; IR (KBr, cm^{-1}) υ_{max}: 3441 and 3352 (NH_2), 2923 (C–H, aliphatic), 2187 (CN); ^{1}H-NMR ($CDCl_3$) δ: 7.29 (dd, J = 2.4 and 8.8 Hz, 1H, arom.), 7.22 (s, 1H, arom.), 7.03 (d, J = 8.4 Hz, 1H, H_5chromene), 6.89 (d, J = 8.8 Hz, 2H, arom.), 6.48 (dd, J = 2.4 and 8.4 Hz, H_6chromene), 6.29 (d, J = 2.4 Hz, 1H, H_8chromene), 6.21 (s, 1H, isoxazole), 4.97 (s, 1H, H_4chromene), 4.70 (s, 2H, NH_2), 3.92 (d, J = 4.4 Hz, 6H, 2 × OCH_3), 2.94 (s, 6H, 2 × CH_3); MS (*m*/*z*, %): 418 [M^+] (20), 354 (20), 280 (10), 214 (100), 189 (34), 165 (72), 150 (18), 79 (20). Anal. calcd. for $C_{23}H_{22}N_4O_4$: C, 66.02; H, 5.30; N, 13.39. Found: C, 66.07; H, 5.05; N, 13.12.

2-Amino-3-cyano-7-dimethylamino-4-(5-(3,4,5-trimethoxyphenyl)isoxazol-3-yl)-4H-chromene ***7o***

Yield: 18%; m.p.: 190–192°C; IR (KBr, cm^{-1}) υ_{max}: 3457 and 3306 (NH_2), 2935 (C–H, aliphatic), 2182 (CN); ^{1}H-NMR ($CDCl_3$) δ: 7.03 (d, J = 8.8 Hz, 1H, H_5chromene), 6.93 (s, 2H, arom.), 6.48 (m,1H, H_6chromene), 6.29 (m, 1H, H_8chromene), 6.25 (s, 1H, isoxazole), 4.97 (s, 1H, H_4chromene), 4.70 (s, 2H, NH_2), 3.90 (s, 6H, 2 × OCH_3), 3.88 (s, 3H, OCH_3), 2.94 (s, 6H, 2 × CH_3); Anal. calcd. for $C_{24}H_{24}N_4O_5$: C, 64.28; H, 5.39; N, 12.49. Found: C, 63.99; H, 5.55; N, 12.22.

Biological activity

Cell lines and cell culture

The synthesized compounds were tested against different human cancer cell lines including MCF-7 (breast cancer), KB (nasopharyngeal epidermoid carcinoma), Hep-G2 (liver carcinoma), MDA-MB-231 (breast cancer), and SKNMC (human neuroblastoma). 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 **7a–o** 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 5 × 10^4 cells/mL. Hundred microliters 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 3 days. The final concentration of DMSO in the highest concentration of the applied compounds was 0.1%. Doxorubicin 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 micro-plate 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.

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