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Synthesis of 4-Aryl-4*H*-Chromene Derivatives with Boric Acid: Evaluation of Their *in vitro* Anti-Angiogenesis Effects and Possible Mechanism of Action

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ABSTRACT

Background: Angiogenesis is the process in which the new blood vessels are formed from pre-existing ones. The development of the vascular system is one of the earliest events in organogenesis. Since angiogenesis is a key process in the promotion of cancer and its metastasis (most common cause of cancer death in human), inhibition of angiogenesis is one of the promising approaches for treatment of tumor growth and metastasis. In this study, several derivatives of 4-aryl-4*H*-chromenes bearing methoxy substituent have been synthesized with boric acid as catalyst and investigated with potential anti-angiogenesis effects as well as anti-angiogenic mechanism. ***Methods:*** Anti-angiogenic and anti-proliferative effects of the prepared compounds checked on three-dimensional culture of human umbilical vein endothelial cells (HUVECs) in collagen matrix and HUVEC proliferation assay as well as matrix metalloproteinase (MMP) gelatinase assay in the endothelial cell-based experimental system. Finally, anti-angiogenic mechanism of the compounds was identified, using gel zymography method. ***Results:*** Among the synthesized compounds 4a and 4h were as the most active in these series. The compound 4a was caused angiogenesis inhibition with complete suppression at 3 µg/ml (8.537 µM) and 4h at 1.6 µg/ml (4.196 µM). ***Conclusion:*** At end, chromene derivatives can serve as the lead molecules for further development of a new class of anti-angiogenesis agents.

Introduction

Cancer is a disease characterized by the uncontrolled growth of abnormal cells. Many studies suggested that once a tumor grows beyond a critical size, which is of approximately 1 mm^3, it has an ability to develop its own blood supply system for the gain off sufficient nutrients and oxygen and the removal of toxic wastes by angiogenesis.[1-3] Angiogenesis, the formation of new blood capillaries from existing vessels, is an important mechanism for supplying nutrients to cells that are distant from existing blood vessels.[4] Angiogenesis is essential in physiological processes (such as growth and development, wound healing and reproduction) and is also involved in pathological conditions such as tumor growth, metastases, and certain chronic diseases.[5] This multi-step process is tightly controlled by a balance of angiogenesis inducers and inhibitors. In pathological conditions (such as tumors), the regulatory mechanisms which "turn off" neovascularization in healthy tissue do not function normally, and a shift in the balance of positive and negative angiogenesis regulators towards the positive molecules is observed.[6] Additionally, angiogenesis is not only responsible for the critical growth of a tumor but also for the metastasis of a tumor.[7-9] Because of these reasons, the effective inhibition of tumor angiogenesis can block tumor progression and growth beyond a critical size or metastasis to other organs.[10]

Green chemistry techniques continue to grow in importance. Alternative processes help to conserve resources and can reduce costs. One of the tools used to combine economic aspects with the environmental ones is the multi component reaction (MCR) strategy, the process consist of two or more synthetic steps which are carried out without isolation of any intermediate, thus reducing time, saving money, energy and raw materials.[11] 4-Aryl-4*H*-chromenes are novel anti-tumor

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agents which synthesize *via* multi component process and induce apoptosis in cancer cells.[12-14] Very recently, anti-angiogenic/proliferative effect of 2-amino-3-cyano-7-(dimethylamino)-4-(2-methoxyphenyl)-4*H*-chromene was confirmed by Mansouri *et al.*[15] As part of our program aimed at developing new selective and environmentally friendly methodologies for the preparation of fine chemicals,[16] we performed the synthesis of other derivatives of 4-aryl-4*H*-chromenes bearing methoxy group at different positions on the phenyl ring through a four-component reaction with boric acid as catalyst (Scheme 1). Then, as a continuation of the efforts to discover and develop 4-aryl-4*H*-chromenes as novel anti-cancer agents, we reported that chromene compounds can inhibit metastasis and, in particular, angiogenesis by using successful approaches such as three-dimensional capillary tube formation as well as matrix metalloproteinase (MMP) gelatinase assay in the endothelial cell-based experimental system. Anti-angiogenic and anti-proliferative effects of chromene compounds **4a-4j** were especially checked on three-dimensional culture of human umbilical vein endothelial cells (HUVECs) in collagen matrix and HUVECs proliferation assay, respectively. Furthermore, possible anti-angiogenic mechanism of the compounds was identified, using gel zymography method.

Materials and Methods

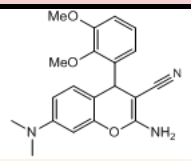
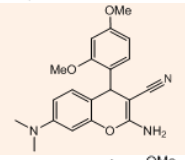
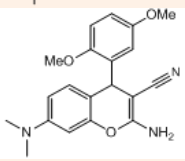
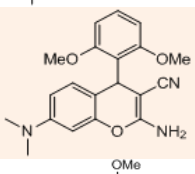
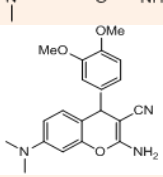
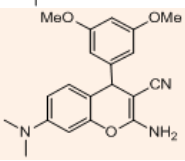
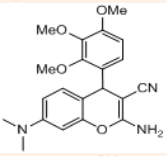
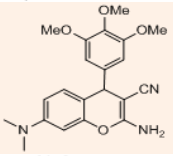
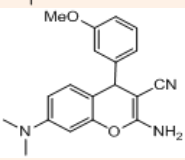
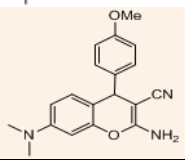
Chemistry

All starting materials, reagents and solvents were purchased from Merck and Aldrich Chemical Companies. All yields refer to isolated yield. The structure of compounds was characterized by IR, ^{1}H NMR spectra and MS. Merck silica gel 60 F254 plates were used for TLC. ^{1}H-NMR spectra were recorded using a Bruker 250 spectrometer and chemical shifts are expressed as (ppm) with tetramethylsilane (TMS) as internal standard using $CDCl_3$ or DMSO-d_6 as solvent. The IR spectra were obtained on a Shimadzu 470 spectrophotometer (potassium bromide disks). The melting point was taken on a Kofler hot stage apparatus and was uncorrected. The mass spectra were run on a Finigan TSQ-70 spectrometer at 70 eV. The purity of the compound was monitored by thin layer chromatography.

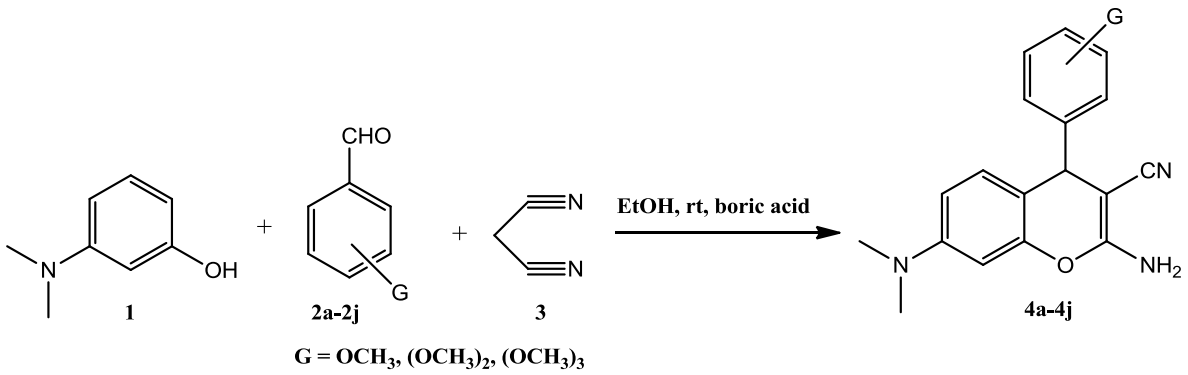
General procedure for the synthesis of chromenes with boric acid as catalyst in ethanol

Chromene derivatives were synthesized by the one-pot four component condensation of an aromatic methoxy aldehyde (1.0 mmol, **2a-2j**), 3-dimethylaminophenol (1.0 mmol, **1**), malononitrile (1.0 mmol, **3**), and boric acid (0.06 g) as catalyst, in ethanol (5 mL) for a designated time listed in Table 1. After completion of the reaction followed by TLC analysis, the reaction mixture was cooled and the precipitated solid was filtered and washed several times with cooled water. The obtained products (**4a-4j**) were crystallized in appropriate solvent (EtOH) and the corresponding chromenes were obtained in 40-75% yields. The spectral data of the compounds **4a-4j** are as followed:

Table 1. Synthesis of chromene compounds **4a-4j** using boric acid at room temperature

Entry	Structure	Yield (%)[a]	Time (h)
4a		46	7
4b		50	8
4c		50	7
4d		45	10
4e		55	7
4f		60	6
4g		70	4
4h		75	4
4i		40	9
4j		40	9

[a] Yield refer to isolated product.



Scheme 1. Reagents and conditions for the synthesis of chromene compounds **4a-4j**.

2-Amino-3-cyano-7-(dimethylamino)-4-(2,3-dimethoxyphenyl)-4H-chromene (4a)

Yield: 46%; m.p. = 158.7-159.8 °C; IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3417 & 3321 (stretch NH, Amine), 3197 (stretch =CH), 2935 & 2835 (stretch CH, Methyl), 2183 (stretch C≡N, Nitrile), 1400 & 1366 (bends CH, Methyl), 1114 (stretch C-N), 1560 (bends NH, Amine), 1477 & 1650 (stretch C=C, Aromatic), 1068 & 1261 (stretch C-O), 748 & 783 (bends oop, =CH); ^{1}H-NMR (DMSO–d_6): δ (ppm) 4.85 (s, H, chromene), 6.23-7.24 (m, H, Aromatic), 2.83 & 3.02 (s, 6H, N-Me), 3.6 & 3.7 (s, 6H, O-Me), 6.7 (s, 2H, Amine); MS (m/z, %): 351 (M^+, 75), 336 (30), 320 (55), 214 (100), 198 (20), 170 (10), 77 (10).

2-Amino-3-cyano-7-(dimethylamino)-4-(2,4-dimethoxyphenyl)-4H-chromene (4b)

Yield: 50%; m.p. = 143.7-144 °C; IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3500 & 3352 (stretch NH, Amine), 3093 (stretch =CH), 2924 & 2850 (stretch CH, Methyl), 2222 (stretch C≡N, Nitrile), 1438 & 1354 (bends CH, Methyl), 1284 (stretch C-N), 1562 (bends NH, Amine), 1562 & 1608 (stretch C=C, Aromatic), 1083 & 1165 (stretch C-O), 798 & 852 (bends oop, =CH); ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.6 (s, H, chromene), 6.44-8.27 (m, H, Aromatic), 3.90 (s, 6H, N-Me), 3.91 (s, 6H, O-Me), 8.17 (s, 2H, Amine); MS (m/z, %): 351 (M^+, 5), 350 (15), 214 (100), 199 (10), 186 (20), 149 (45), 121 (35), 77 (15).

2-Amino-3-cyano-7-(dimethylamino)-4-(2,5-dimethoxyphenyl)-4H-chromene (4c)

Yield: 50%; m.p. = 108.4-108.5 °C; IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3300 & 3400 (stretch NH, Amine), 2924 & 2854 (stretch CH, Methyl), 2341 (stretch C≡N, Nitrile), 1450 & 1380 (bends CH, Methyl), 1238 (stretch C-N), 1560 (bends NH, Amine), 1496 & 1654 (stretch C=C, Aromatic), 1045 & 1083 (stretch C-O), 798 & 871 (bends oop, =CH); ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.58 (s, H, chromene), 6.91-7.74 (m, H, Aromatic), 3.82 (s, 6H, N-Me), 3.88 (s, 6H, O-Me), 8.29 (s, 2H, Amine); MS (m/z, %): 351 (M^+, 25), 350 (100), 322 (45), 307 (15), 214 (95), 199 (98), 170 (35), 141 (38), 86 (90), 77 (50).

2-Amino-3-cyano-7-(dimethylamino)-4-(2,6-Dimethoxyphenyl)-4H-chromene (4d)

Yield: 45%; m.p. = 151 °C; IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3400 (stretch NH, Amine), 3000 (stretch =CH), 2951 & 2846 (stretch CH, Methyl), 2225 (stretch C≡N, Nitrile), 1570 (bends NH, Amine), 1481 & 1600 (stretch C=C, Aromatic), 1435 & 1357 (bends CH, Methyl), 1215 (stretch C-N), 1111 & 1269 (stretch C-O), 725 & 790 (bends oop, =CH), ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.60 (s, H, chromene), 3.91 (s, 6H, N-Me), 3.98 (s, 6H, O-Me), 6.56-7.48 (m, H, Aromatic), 7.99 (s, 2H, Amine); MS (m/z, %): 351 (M^+, 10), 350 (25), 336 (7), 320 (9), 306 (5), 214 (100), 199 (15), 170 (54), 149 (75), 141 (55), 136 (15), 77 (60).

2-Amino-3-cyano-7-(dimethylamino)-4-(3,4-dimethoxyphenyl)-4H-chromene (4e)

Yield: 55%; m.p. = 147-147.7 °C; IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3448 & 3500 (stretch NH, Amine), 3100 (stretch =CH), 2935 & 2850 (stretch CH, Methyl), 2222 (stretch C≡N, Nitrile), 1571 (bends NH, Amine), 1512 & 1423 (stretch C=C, Aromatic), 1469 (bends CH, Methyl), 1276 (stretch C-N), 1018 & 1145 (stretch C-O), 821 & 848 (bends oop, =CH); ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.66 (s, H, chromene), 6.96-7.68 (m, H, Aromatic), 3.94 (s, 6H, N-Me), 3.99 (s, 6H, O-Me), 7.65 (s, 2H, Amine); MS (m/z, %): 351 (M^+, 10), 350 (27), 336 (5), 214 (100), 199 (27), 171 (30), 141 (20), 77 (22).

2-Amino-3-cyano-7-(dimethylamino)-4-(3,5-dimethoxyphenyl)-4H-chromene (4f)

Yield: 60%; m.p. = 96 °C; IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3433 & 3500 (stretch NH, Amine), 3032 (stretch =CH), 2924 & 2846 (stretch CH, Methyl), 2229 (stretch C≡N, Nitrile), 1581 (bends NH, Amine), 1458 & 1604 (stretch C=C, Aromatic), 1427 (bends CH, Methyl), 1311 (stretch C-N), 1064 & 1207 (stretch C-O), 675 & 829 (bends oop, =CH); ^{1}H-NMR

($CDCl_3$): δ (ppm) 1.59 (s, H, chromene), 6.69-7.68 (m, H, Aromatic), 3.84 (s, 6H, N-Me), 3.94 (s, 6H, O-Me), 7.68 (s, 2H, Amine); MS (m/z, %): 351 (M^+, 20), 350 (100), 336 (15), 322 (20), 307 (5), 214 (45), 186 (30), 170 (20), 141 (22), 86 (48), 77 (22).

2-Amino-3-cyano-7-(dimethylamino)-4-(2,3,4-trimethoxyphenyl)-4H-chromene (4g)

Yield: 70%; m.p. = 104 °C; IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3371 & 3400 (stretch NH, Amine), 3090 (stretch =CH), 2951 & 2839 (stretch CH, Methyl), 2222 (stretch C≡N, Nitrile), 1573 (bends NH, Amine), 1470 & 1590 (stretch C=C, Aromatic), 1458 (bends CH, Methyl), 1300 (stretch C-N), 1099 (stretch C-O), 802 & 898 (bends oop, =CH); ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.57 (s, H, chromene), 6.77-8.107 (m, H, Aromatic), 3.86 (s, 6H, N-Me), 3.97 (s, 9H, O-Me), 8.12 (s, 2H, Amine); MS (m/z, %): 381 (M^+, 25), 380 (100), 366 (8), 350 (20), 316 (30), 244 (95), 229 (50), 186 (35), 171 (20), 77 (15).

2-Amino-3-cyano-7-(dimethylamino)-4-(3,4,5-trimethoxyphenyl)-4H-chromene (4h)

Yield: 75%; m.p. = 187 °C; IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3406 & 3332 (stretch NH, Amine), 3200 (stretch =CH), 2931 & 2850 (stretch CH, Methyl), 2191 (stretch C≡N, Nitrile), 1593 (bends NH, Amine), 1462 & 1650 (stretch C=C, Aromatic), 1450 (bends CH, Methyl), 1234 (stretch C-N), 1122 & 1327 (stretch C-O), 798 & 817 (bends oop, =CH); ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.63 (s, H, chromene), 6.28-6.83 (m, H, Aromatic), 2.93 (s, 6H, N-Me), 3.81 (s, 9H, O-Me), 8.5 (s, 2H, Amine); MS (m/z, %): 381 (M^+, 28), 366 (3), 350 (4), 214 (100), 198 (15), 186 (3), 170 (10).

2-Amino-3-cyano-7-(dimethylamino)-4-(3-methoxyphenyl)-4H-chromene (4i)

Yield: 40%; m.p. = 106.8-107 °C; IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3400 & 3500 (stretch NH, Amine), 3039 (stretch =CH), 2943 & 2843 (stretch CH, Methyl), 2230 (stretch C≡N, Nitrile), 1496 & 1600 (stretch C=C, Aromatic), 1667-2000 (overtone, Aromatic), 1570 (bends NH, Amine), 1462 & 1373 (bends CH, Methyl), 1161 (stretch C-N), 1041 & 1280 (stretch C-O), 683 & 780 & 864 (bends oop, =CH); ^{1}H-NMR (DMSO–d_6): δ (ppm) 3.83 (s, H, chromene), 7.25-7.54 (m, H, Aromatic), 3.53 & 3.6 (s, 6H, N-Me), 3.7 (s, 3H, O-Me), 8.45 (s, 2H, Amine); MS (m/z, %): 321 (M^+, 20), 184 (100), 156 (30), 141 (20), 127 (40), 114 (45), 88 (15), 75 (10).

2-Amino-3-cyano-7-(dimethylamino)-4-(4-methoxyphenyl)-4H-chromene (4j)

Yield: 40%; m.p. = 115.2-115.5 °C; IR (KBr, cm^{-1}): $\bar{\upsilon}$ 3400 & 3500 (stretch NH, Amine), 3028 (stretch =CH), 2924 & 2850 (stretch CH, Methyl), 2222 (stretch C≡N, Nitrile), 1512 & 1604 (stretch C=C, Aromatic), 1573 (bends NH, Amine), 1427 & 1370 (bends CH, Methyl), 1184 (stretch C-N), 1083 & 1280 (stretch C-O), 833 (bends oop, =CH), ^{1}H-NMR ($CDCl_3$): δ (ppm) 1.56 (s, H, chromene), 7-7.92 (m, H, Aromatic), 3.91 (s, 6H, N-Me), 3.91 (s, 3H, O-Me), 7.92 (s, H, Amine); MS (m/z, %): 321 (M^+, 20), 184 (100), 141 (27), 127 (10), 114 (40).

Cell isolation/culture

Human umbilical vein endothelial cells were obtained from human umbilical vein of newborns using actinidin digestion method according to Mostafaie et al.[17] In this way, written informed consent was obtained from all participating parents after careful explanation of the study. The isolated endothelial cells were started from frozen stock and grown on tissue culture-treated plastic in endothelial cell basal medium supplemented with 10% heat-inactivated FBS and maintained at 37 °C with 5% CO_2 until 90% confluent.

Cytotoxicity assay

To determining maximum non-toxic (and cytotoxic) concentrations of each test compound, different concentrations of compound **4a-j** were added to medium containing confluent HUVEC. After 48 hours of incubation, the effect of compounds on cell viability was determined by trypan blue exclusion as well as lactate dehydrogenase (LDH) assays.[18,19] The absorbance of converted dye in LDH assay was measured at wavelength of 490 nm with background subtraction at 630 nm. The absorbance of treated cells was compared with the absorbance of the controls, which cells were exposed only to the vehicle (DMSO) and were considered as 100% viability value. The final concentration of DMSO in the test medium and controls was less than 1% throughout this investigation. Each concentration was tested in three independent experiments.

Anti-proliferation assay

Anti-proliferation assay was performed on HUVEC, in MCDB131 medium supplemented with 10% FBS. Exponentially, growing cells were seeded in round-bottomed plates and allowed to attach overnight. After 24 h incubation at 37 °C and 5% CO_2, different amounts of the test compounds **4a-j** were added, and the cells were exposed to drugs for additional 3 days. The cells were then harvested by trypsinization and counted against control wells by a coulter counter. The IC_{50} values were calculated and represent the concentration of drug causing 50% inhibition in cell proliferation.[15-24]

HUVEC capillary tube formation in three-dimensional collagen gel and evaluation of angiogenesis in vitro

HUVEC cells were grown in medium supplemented with 10% FBS at 37 °C and 5% CO_2 and after 3-5 passages were used for this experiment. The cells

were mixed with sterilized cytodex-3 micro-carriers coated beads with gelatin, at a ratio of 30 HUVEC cells per bead in one ml of medium supplemented with 10% heat-inactivated FBS. Beads with cells were shaken gently every 20 min for 4 h at 37 ^{o}C and 5% CO_2. The mixture were transferred to 4 wells of a 24-well tissue culture plate and left for 12-16 h in one ml of medium at 37 ^{o}C and 5% CO_2. The following day, cell-coated beads were re-suspended (cultured) in type I collagen matrix and, for gel formation, were placed in 37 ^{o}C/5% CO_2 incubator, as described above. After collagen gel formation, MCDB-131 medium was added to each well. In order to study anti-tubulogenesis effect of the test compounds, different concentrations of the compounds were added to the wells. The plates were incubated for three days and anti-angiogenic effects of the test compounds on the formation of capillary-like structures were monitored under microscope, daily, images were captured and then the number of structures was quantified by counting all branches in three random fields from each well.[15,24]

Gelatin Zymography

Effect of test compound on the enzymatic activity of MMP-2 and MMP-9 was assessed using gelatin zymography.[20] Confluent HUVECs were isolated and immediately incubated in the absence of serum (FBS) with different concentrations of the test compounds for 16 hours. Firstly, protein content of the serum free supernatant media from HUVECs treated with test compound was measured by the method of Bradford.[21] Then, the supernatant of treated and control wells were mixed with sample buffer and loaded onto a 7.5% polyacrylamide gel containing 2 mg/ml gelatin under non-reducing condition. Following electrophoresis, gels were washed twice with washing buffer containing 2.5% Triton X-100 for one hour at room temperature (to remove SDS) and then incubated for 16 h at 37 ^{o}C in the activation buffer (50 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$) for development of enzyme activity bands. Thereafter, the gels were stained with Coomassie brilliant blue R-250 in a 30% methanol and 10% glacial acetic acid for 2 h and distained in methanol-acetic acid mixed solution. The gelatinolytic activities of MMPS were detected as clear/transparent bands against the background of coomassie brilliant blue stained gelatin. In a series of independent experiments, different concentrations of the test compound were added at (SDS to Triton) exchanging step for evaluating their direct inhibitory effects on MMP gelatinase activity.

Results and Discussion

Generality and optimization of reaction conditions

Although, the reported methods for the synthesis of chromene derivatives are effective, they are confronted with certain drawbacks of environment compatibility by the use of toxic and expensive catalysts, which also lack recyclability. While boric acid is eco-friendly and inexpensive catalyst. In this approach we used ethanol instead of large amounts of organic solvents, work-up is simplified, and the reaction times considerably decreased. In this reaction, the one-pot synthesis was optimized by a mixture of 3-dimethylaminophenol (**1**) (1.0 mmol), aromatic aldehyde (**2a-2j**) (1.0 mmol), and malononitrile (**3**) (1.0 mmol) in the presence of boric acid (0.06 g) in ethanol (5 mL) and was stirred at 25 ^{o}C for time specified in Table 1. The experimental procedure is very simple and convenient, and under the reaction conditions used, can tolerate a variety of methoxy. Simply by adding catalyst to a mixture of reactants, rapid and convenient condensation is achieved at 25 ^{o}C in ethanol. After the completion of the reaction (monitored by TLC), the residue was filtered and washed with cooled water then was purified by recrystallization from ethanol to produce the desired solid and confirmed by physical and spectral data.

The maximum non-toxic concentration of the test compounds obtained by addition of the compounds **4a-4j** to culture medium containing confluent HUVECs. Since ideal anti-angiogenesis drugs are expected to have effect against pathogenic angiogenesis without producing cytotoxicity in the target cells (HUVEC), and because, among test chromene compounds, only compounds **4a** and **4h** have significant anti-angiogenic (and anti-proliferative) effect on HUVECs at non-toxic concentrations (compound **4a** was not cytotoxic up to 6 µg/ml and compound **4h** up to 4 µg/ml as assessed by LDH cytotoxicity assays). Thus, these compounds were selected to study in details and the other compounds (which had not only highly cytotoxic concentration but also no anti-proliferative/angiogenic activity at their non-toxic concentration ranges) were excluded from further studies.

Proliferation of endothelial cells is an absolute requirement for angiogenesis. The compounds **4a** and **4h** induced significant decrease in the proliferation of HUVEC in a dose-dependent manner (Figure 1). Furthermore, this inhibitory effect did not result of cytotoxic effect, as assessed by LDH cytotoxicity assays, compared with controls. Taking the results into account, it can be concluded that endothelial cell proliferation, as major prerequisite of angiogenesis, was suppressed in the presence of less than 6 µg/ml (17.074 µM) of compound **4a** and less than 4 µg/ml (10.486 µM) of compound **4h**.

These compounds were also able to inhibit completely angiogenesis in HUVEC capillary tube formation model in a dose-dependent manner (Figure 2). The ability for endothelial cells to form tubular structures on cytodex matrix, offers an *in vitro* model of angiogenesis. In order to evaluate the

possible effect of selected 4-aryl-4H-chromenes as angiogenesis inhibitors, increasing concentrations of compounds **4a** and **4h** were added to endothelial cells. A dose-dependent anti-angiogenic behavior was observed and representative results are shown in Figure 3 and Figure 4. As it is evident from figures, compound **4a** was causing angiogenesis inhibition with complete suppression at 3 µg/ml (8.537 µM) and compound **4h** at 1.6 µg/ml (4.196 µM).

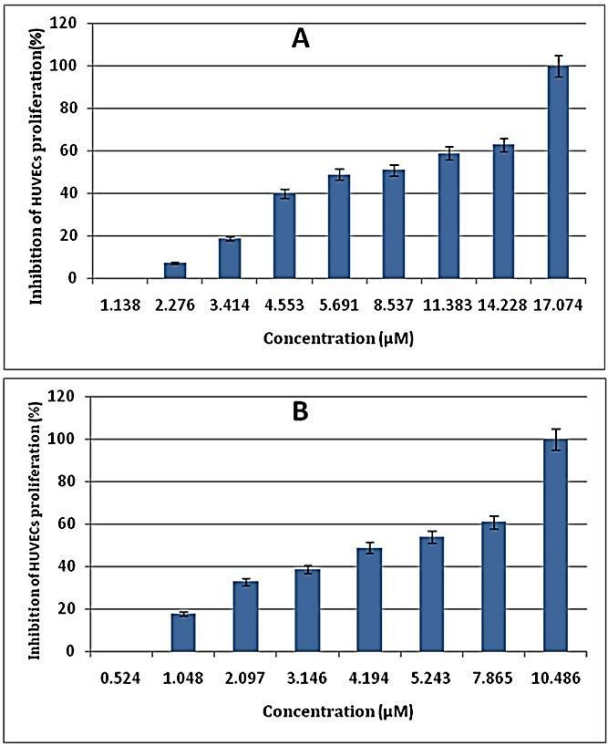


Figure 1. Inhibition of HUVECs proliferation by compounds **4a** (A) and **4h** (B).

Angiogenesis is tightly regulated by complex system of pro- and anti-angiogenic factors including growth factors and Matrix Metalloproteinases (MMPs).[15] It is initiated by the release of proteases that allow degradation of the basement membrane and followed by proliferation/migration of endothelial cells. Once a tumor is established, interactions between stromal, tumor, and endothelial cells will trigger secretion/activation of various MMPs followed by degradation and invasion of the extracellular matrix (ECM) which permits tumor cell motility (within ECM) and metastases as well as facilitates budding of new blood vessels. To date, more than 25 human MMPs are known. These MMPs are classified into collagenases, gelatinases, stromelysins, matrilysins, and membrane-type MMPs.[15,22,23] Metalloproteinases have attracted significant interest not only because they degrade the ECM molecules but because they also regulate growth factor signaling, cell adhesion, and release pro- and anti-angiogenic fragments of ECM proteins. Furthermore, most MMPs are secreted as zymogenes upon cytokine stimulation and require extracellular proteolytic activation. MMP-2 (gelatinase A, EC 3.4.24.24) and MMP-9 (gelatinase B, EC 3.4.24.35) have been extensively studied owing to their consistent association with tumor invasion and metastasis. In this way inhibition of expression, conversion and/or biological (enzymatic) function of these proteases may block angiogenesis both *in vivo* and *in vitro*.[22,23] There is a possibility that test compounds (**4a** and **4h**) exert their activity via inhibition of MMP function. In this frame and to test this possibility, we examined the expression, conversion, or enzymatic function of MMPs in the presence or the absence of these chromene compounds. As shown by Figure 5, The MMP-9 and MMP-2 activity were suppressed in a dose-dependent manner, with an increase of compound **4a** and compound **4h** concentrations. Complete suppression of MMP-9 and MMP-2 expression/activity was obtained in the presence of moderate concentration of compound **4h** (400 ng/ml-1.6 µg/ml) and compound **4a** (800 ng/ml-3 µg/ml). These results suggested that the tested inhibitors are involved in the expression/activity of both MMP-9 and MMP-2.

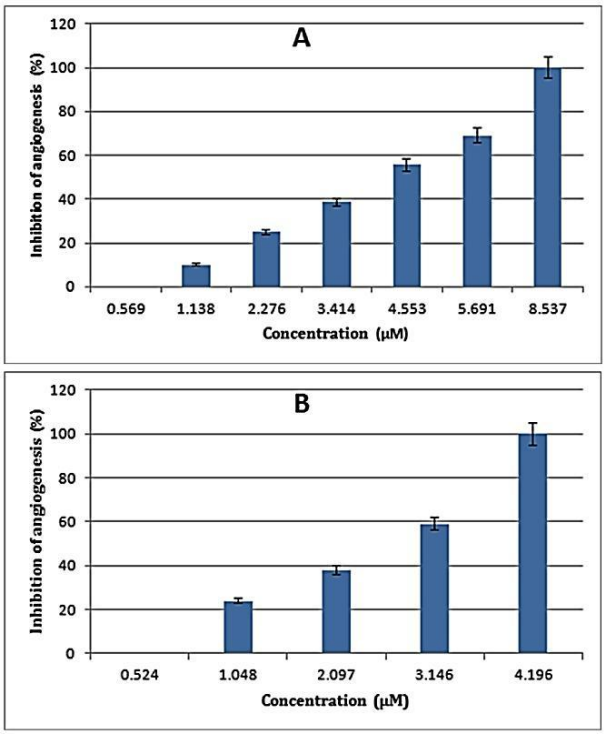


Figure 2. Inhibition of angiogenesis in HUVEC capillary tube formation model by compounds **4a** (A) and **4h** (B).

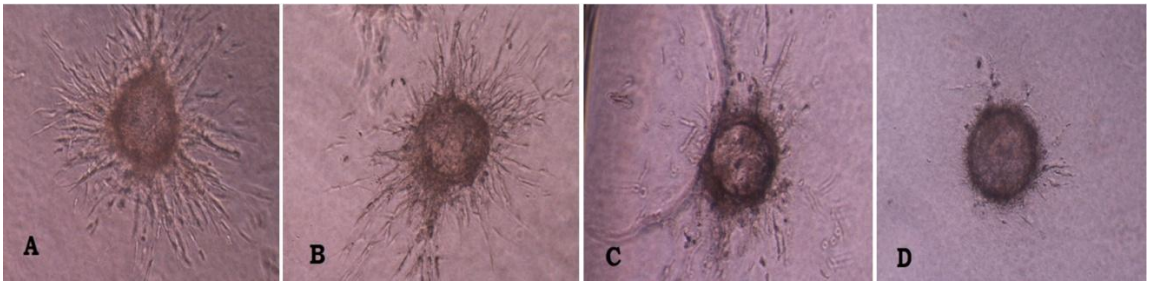


Figure 3. Anti-angiogenic activity of 2-amino-3-cyano-7-(dimethylamino)-4-(2,3-dimethoxyphenyl)-4*H*-chromene (**compound 4a**) on *in vitro* HUVEC capillary tube formation. Spontaneous formation of capillary-like structures by HUVECs on ''dextran-coated cytodex-3 microcarriers'' was used to assess anti-angiogenic potential**. A)** Angiogenesis of endothelial cells in the untreated wells (negative control). The endothelial cell attached to particles has been migrated through the collagen matrix. **B-D)** Inhibition of angiogenesis of the endothelial cells treated by different (2.276, 5.691 and 8.537 µM) concentrations of compound **4a**.

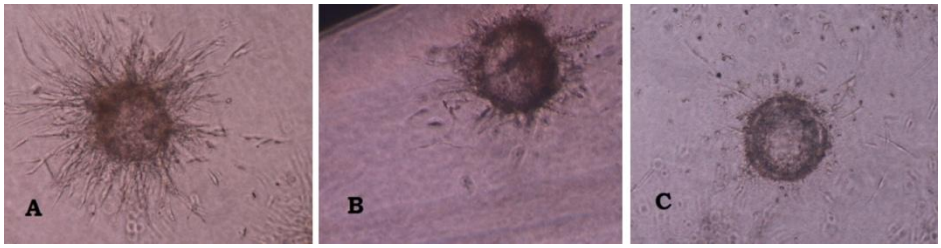


Figure 4. Anti-angiogenic activity of 2-amino-3-cyano-7-(dimethylamino)-4-(3,4,5-trimethoxyphenyl)-4*H*-chromene (**compound 4h**) on in vitro HUVEC capillary tube formation. **A)** Angiogenesis of endothelial cells in the untreated wells (negative control). Inhibition of angiogenesis of the endothelial cells treated by **B)** 3.146 µM **C)** 4.194 µM of compound **4h**.

Furthermore, whether these observations are due to enzyme- or gene expression-inhibitory properties of these compounds is in process through an independent project. In this way, study of MMP expression under the effect of compounds, using RT-PCR technique, structure–activity relationship studies, are in process. In addition, it has been previously observed that proteolytic activity of some MMPs (MMP-2 and MMP-9) decreases cancer cell apoptosis via different mechanisms. Then, inhibition of MMPs, in this context, may also have an additional apoptosis-inducing effect regarding to abnormal inhibition of apoptosis by cancer cells. Taking the results into account, anti-angiogenic activity of selected test compounds is not only associated with a decrease in endothelial cell proliferation but also can be attributed to inhibition of MMP activity and/or interference of cytoskeleton organization, which are known to play important roles in cell locomotion and capillary tube formation.[15,21] There is a possibility that chromene compounds both induces apoptosis in cancer cells and inhibit EC angiogenesis via targeting tubulin/cytoskeleton (as well as MMPs) system. In order to test these possibilities, extensive experimental works are also needed. Moreover, since pathogenic angiogenesis (during tumor and/or obesity neovascularization) mainly encompasses microvascular level of blood vessel system,[24] we have also planned to test potent compounds using microvascular ECs.

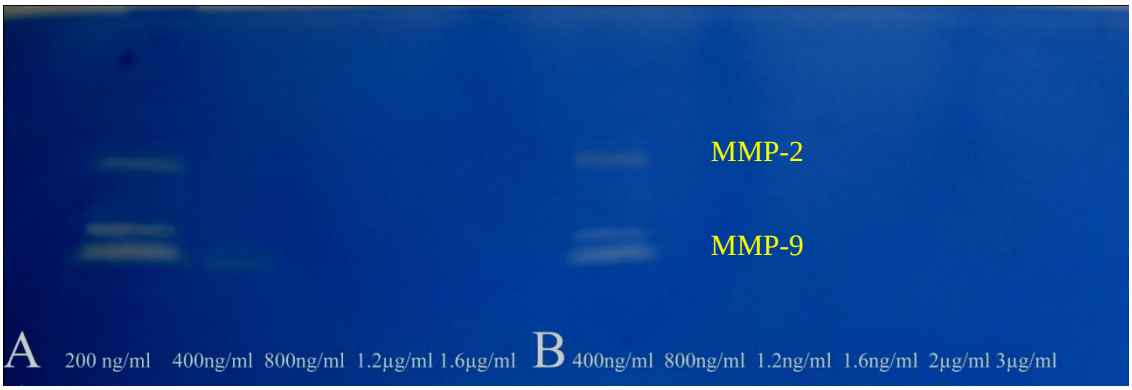


Figure 5. Gelatin zymography assay of extracellular MMP activity of HUVECs treated with increasing concentrations of **A)** compound **4h** and **B)** compound **4a**. Complete suppression of MMP-9 and MMP-2 expression/activity was obtained in the presence of moderate concentration of compound **4h** (400 ng/ml-1.6 µg/ml) and compound **4a** (800 ng/ml-3 µg/ml).

Conclusion

Since angiogenesis is a key process in the promotion of cancer and its metastasis, its inhibition is one of the promising approaches for treatment of tumor growth and metastasis. Two synthesized compounds; 2-amino-3-cyano-7-(dimethylamino)-4-(2,3-dimethoxyphenyl)-4*H*-chromene and 2-amino-3-cyano-7-(dimethylamino)-4-(3,4,5-trimethoxyphenyl)-4*H*-chromene, displayed anti-cell proliferation and inhibiting effect of MMP-2 and MMP-9 and may act as apoptosis inducer in cancer cells/angiogenesis inhibitor in ECs via targeting tubulin/cytoskeleton (as well as MMPs) system, thus they can be good candidates for inhibition of angiogenesis.

Acknowledgments

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