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4-Methylumbelliferone Inhibits Angiogenesis in Vitro and in Vivo

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ABSTRACT: 4-Methylumbelliferone (4-MU) is a hyaluronic acid biosynthesis inhibitor with antitumoral and antimetastatic effects. The objective of the present study was to determine the potential of 4-MU as an antiangiogenic compound. To fulfill this aim, cultured endothelial cells were used to perform an array of in vitro assays, as well as two different in vivo angiogenesis assays. This study demonstrates that, in fact, 4-MU behaves as a new inhibitor of both in vitro and in vivo angiogenesis. In vitro, 4-MU affects several key steps of angiogenesis, including endothelial cell proliferation, adhesion, tube formation, and extracellular matrix remodeling. Half-maximal inhibitory concentrations (IC_{50}) values in the proliferation assay were 0.65 ± 0.04 and 0.37 ± 0.03 mM for HMEC and RF-24 endothelial cells, respectively. 4-MU (2 mM) treatment for 24 h induced apoptosis in 13% of HMEC and 5% of RF-24 cells. The number of adherent endothelial cells decreased by >20% after 24 h of treatment with 1 mM 4-MU. Minimal inhibitory concentrations in the tube formation assay were 2 and 0.5 mM 4-MU for HMEC and RF-24, respectively. Matrix metalloproteinase-2 expression was differentially altered upon 4-MU treatment in both tested endothelial cell lines. Taken together, the results suggest that 4-MU may have potential as a new candidate multitargeted bioactive compound for antiangiogenic therapy.

KEYWORDS: *4-methylumbelliferone, angiogenesis, endothelial cells*

INTRODUCTION

Miguel de Cervantes's *D. Quixote* mentions a so-called *Fierabras' balsam*, a marvelous, magical, miraculous remedy for all kind of illnesses. Past and present history of the pharma industry and related scientific research are pervaded by the search for new drugs with the potential to become actual Fierabras' balsams. The identification of new multitargeted drugs could be particularly relevant for tumor angiogenesis,[1] because preclinical and clinical results seem to indicate that a multitargeted approach could be expected to produce better results in antiangiogenic therapy.[2] Angiogenesis is the generation of new capillaries by sprouting of pre-existing microvessels. It is a very important biological process in both health and disease.[3,4] In health, vessel proliferation is tightly regulated, occurring only for embryonic development, endometrial regulation, reproductive cycle, and wound repair. On the contrary, a persistent and deregulated angiogenesis is related to many diseases, currently known as angiogenesis-dependent diseases. This is the case of proliferative retinopathies, psoriasis, and rheumatoid arthritis. Furthermore, angiogenesis is essential for tumor progression and is described as one of the hallmarks of cancer.[5] Because angiogenesis is involved in very different diseases, it has been suggested as an organizing principle in drug discovery.[6] Therefore, the development of any therapeutic strategy for an angiogenesis-dependent disease could help the development of therapeutic strategies for other angiogenesis-dependent diseases. On the basis of this principle, the search for modulatory compounds of angiogenesis is currently a very hot topic.[4,7,8] Our group is actively involved in the identification and characterization of new natural bioactive compounds with multitargeted anti-angiogenic effects.[9−16]

4-Methylumbelliferone (4-MU, see Figure 1) is a hydroxycumarin obtained from umbelliferous plants (Apiaceae), including anise, cumin, parsley, and dill, among others. This compound is described as a hyaluronic acid biosynthesis specific inhibitor, because it is able to inhibit hyaluronan synthases 2 and 3.[17,18] Furthermore, glucuronidation of 4-MU can deplete the pool of UDP-glucuronic acid, which is necessary for hyaluronic acid synthesis.[18−21] Along with these properties, 4-MU has been shown to have antitumoral and antimetastatic potential, being able to affect proliferation, adhesion, motility and invasiveness of different cultured cells.[22−28] All of these reported effects of 4-MU have been observed when used in the millimolar−submillimolar range. Some of these features are shared with previously described antiangiogenic compounds, but up to now no reported study has determined whether 4-MU can behave as an antiangiogenic compound or not. The objective of the present study was to determine for the first time the potential antiangiogenic effects of 4-MU. To fulfill this aim, we used both macrovascular (RF-24) and microvascular (HMEC) endothelial cells to perform an array of in vitro assays, allowing for testing the effects of 4-MU on key steps of the angiogenic process. Furthermore, we also

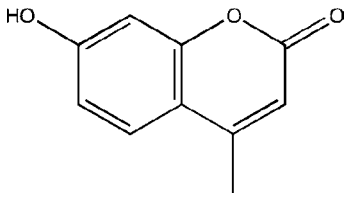

Figure 1. Chemical structure of 4-methylumbelliferone.

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carried out two different in vivo angiogenesis assays. Results here presented are the first experimental evidence showing that 4-MU has the potential to behave as a new inhibitor of key steps of angiogenesis.

MATERIALS AND METHODS

Chemicals. Matrigel was supplied by BD, and calcein-AM was purchased from Molecular Probes, currently a branch of Invitrogen. All other chemicals, included 4-MU, were supplied by Sigma-Aldrich.

Cell Cultures. Two immortalized human endothelial cell lines kindly supplied by Dr. Arjan W. Griffioen (Maastrich University, The Netherlands) were used during this study: human umbilical vein endothelial cells (RF-24) and human microvascular endothelial cells (HMEC). Thrsr cell lines have been previously characterized.[29] Both immortalized human endothelial cell lines were grown in RPMI-1640 medium supplemented with glutamine (2 mM), penicillin (50 IU/mL), streptomycin (50 mg/L), amphotericin (1.25 mg/L), 10% fetal bovine serum, and 10% human serum.

MTT Cell Growth Assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay in 96-well microplates was used. The assay is dependent on the reduction of MTT by mitochondrial dehydrogenases of viable cells to a blue formazan product, which can be measured spectrophotometrically. Endothelial cells (2.5×10^3 cells in a total volume of 100 μL of complete medium) were incubated in each well with serial dilutions of 4-MU. After 3 days of incubation in the dark (37 °C, 5% CO_2 in a humid atmosphere), 10 μL of MTT (5 mg/mL in PBS) was added to each well, and the plate was incubated for further a 4 h (37 °C). The formazan was dissolved in 150 μL of 0.04 N HCl−2 propanol, and samples were spectrophotometrically measured at 550 nm. All determinations were carried out in quadruplicate, and at least three independent experiments were carried out. IC_{50} values were calculated as those concentrations of compound yielding 50% cell survival, taking the values obtained for control as 100%.

The rest of the in vitro assays used in this study were carried out under conditions (4-MU concentrations and duration of treatments) that produced no cytotoxic effect on cells. This lack of cytotoxitiy was determined by modified MTT survival assays, that is, MMT assays carried out at the same 4-MU concentrations and duration of treatments used in the rest of the in vitro assays described in this study (routinary control results not shown).

Cell Cycle Analysis by Flow Cytometry. Cells at >80% of confluence in 6-well plates were treated with different concentrations (0−2 mM) of 4-MU for 24 h. After incubation, attached and unattached 4-MU-treated and control cells were harvested, washed (PBS), and fixed (70% ethanol, 1 h on ice). Pelleted cells were incubated (1 h protected from light) with RNase A (0.1 mg/mL) and propidium iodide (40 μg/mL) during 1 h of shaking and protected from light. Percentages of sub-G1, G1, and G2/M populations were determined using a Dako MoFlow cytometer and its software, Summit 4.3.

Assay for Tube Formation on Matrigel by Endothelial Cells. Matrigel (50 μL of about 10.5 mg/mL) at 4 °C was used to coat each well of a 96-well plate and allowed to polymerize at 37 °C for a minimum of 30 min. A total of 5×10^4 cells were added with 200 μL of medium. Finally, different amounts of 4-MU were added and incubated at 37 °C in a humidified chamber with 5% CO_2. After incubation of 5 h in the dark, cultures were observed and photographed with a Nikon inverted microscope DIAPHOT-TMD (Nikon Corp., Tokyo, Japan). Each concentration was tested in triplicate. "Tubular" structures were counted using image analysis tools. Each coherent, noninterrupted, closed network was counted as a "tube".

Adhesion Assay. The wells of 24-well plates were covered with 1 mL of 0.5% gelatin and maintained at 37 °C for 1 h. Then, the leftover ungelled gelatin was aspirated, 0.3 mL of 7.5% bovine serum albumin was added to each well, and plates were maintained at 37 °C for another hour, and afterward they were withdrawn.

Endothelial cells were incubated at 37 °C in the absence or presence of the tested concentrations of 4-MU for 24 h. Calcein-AM (1 mg/mL) was added to cells 2 h prior to the end of incubation. After incubation, cells were washed twice with PBS. Cells were suspended at 3×10^5 cells/mL in complete culture medium, and 0.3 mL of cell suspension was added to each well. After 1 h of incubation at 37 °C, wells were gently washed three times with PBS. Finally, cells that remained attached were counted and photographed in an inverted epifluorescence Nikon microscope.

Scratch Wounding Migration Assay. The migratory activity of endothelial cells was assessed using a "wound-healing" migration assay. Confluent monolayers in 6-well plates were wounded with pipet tips following two perpendicular diameters, giving rise to two acellular 1-mm-wide lanes per well. After washing, cells were supplied with 1.5 mL of complete medium in the absence (controls) or presence of different concentrations (0.25−2 mM) of 4-MU. Photographs were taken at different times of incubation (after 7 and 24 h) in the dark. The amount of migration at 7 and 24 h was determined by image analysis in both control and treated wells.

Zymographic Assay for MMP-2. To prepare endothelial cell conditioned media and cell lysates, both HMEC and RF-24 cells were grown in 6-well plates. When cell cultures were almost at confluence, medium was withdrawn, cells were washed twice with phosphate-buffered saline (PBS), and each well received 1.5 mL of DMEM/0.1% BSA containing 200 KIU aprotinin/mL. Additionally, some wells received 4-MU at the concentrations mentioned under Results. After 24 h of incubation, conditioned media were collected. Cells were washed twice with PBS and harvested by scraping into 0.5 mL of 0.2% Triton X-100 in 0.1 M Tris-HCl containing 200 KIU aprotinin/mL. Media and cell lysates were centrifuged at 1000*g* and 4 °C for 20 min. Afterward, the supernatants were collected and used for zymography. Duplicates were used to determine cell number with a Coulter counter. The gelatinolytic activity of matrix metalloproteinase-2 (MMP-2) delivered to the conditioned media and present in cell extracts was detected in gelatinograms. Aliquots of conditioned media and cell lysates normalized for equal cell numbers were subjected to nonreducing SDS-PAGE as above but with gelatin (1 mg/mL) added to the 10% resolving gel. After electrophoresis, gels were washed twice with 50 mM Tris-HCl, pH 7.4, supplemented with 2% Triton X-100, and twice with 50 mM Tris-HCl, pH 7.4. Each wash with continuous shaking lasted for 10 min. Then, gels were incubated overnight at 37 °C and immersed in a substrate buffer (50 mM Tris-HCl, pH 7.4, supplemented with 1% Triton X-100, 5 mM $CaCl_2$, and 0.02% Na_3N). Finally, the gels were stained with Coomassie blue R-250, and the bands of gelatinase activity could be detected as nonstained bands in a dark, stained background.

In Vivo Chorioallantoic Membrane (CAM) Assay. Fertilized chick eggs were incubated horizontally at 38 °C in a humidified incubator, windowed by day 3 of incubation and processed by day 8. The indicated amount of 4-MU was added to a 1.2% solution of methylcellulose in water, and 10 μL drops of this solution were allowed to dry on a Teflon-coated surface in a laminar flow hood. Then, the methylcellulose disks were implanted on the CAM, and the eggs were sealed with adhesive tape and returned to the incubator for 48 h. Negative controls were always made with DMSO mixed with the methylcellulose. After the reincubation, the CAM was examined under a stereomicroscope. The assay was scored as positive when two independent observers reported a significant reduction of vessels in the treated area.

In Vivo Angiogenesis Assay Using a Model of Zebrafish. A transgenic line of zebrafish (*Danio rerio*) exhibiting fluorescent blood vessels, thanks to the expression of a choral green fluorescent protein (G-RCFP) controlled by a promoter for VEGF-R2,[30] was used. One day after mating, eggs were transferred to a Petri dish, where they were treated with lye diluted to 0.5% in water for 90 s. Then, they were washed three times for 3 min with water and maintained for 24 h at 28.5 °C. After this new incubation, chorion was retired, and larvae were transferred to 96-well plates (a larva per well) with 0.1 mL of water per well in the presence of the indicated concentration of 4-MU. After an additional 24 h of incubation at 28.5 °C, effects on blood

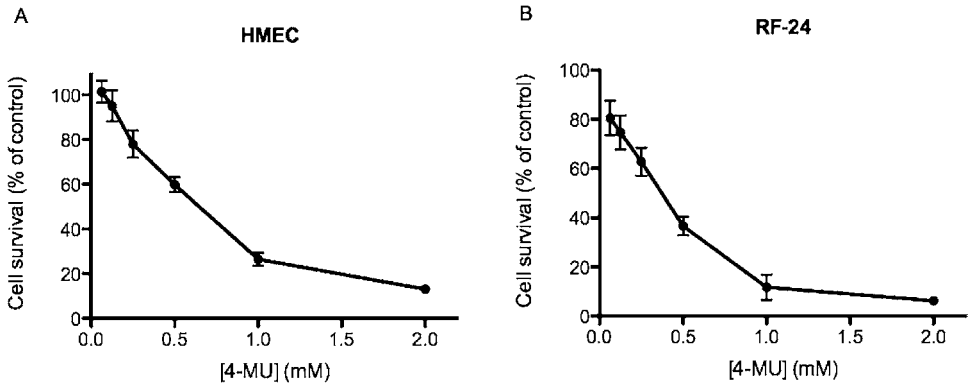

Figure 2. Effects of 4-methylumbelliferone (4-MU) on endothelial cell proliferation as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Experiments were carried out as described under Materials and Methods. Data represent the mean ± SD for three independent experiments (each one with four replicates of each tested concentration).

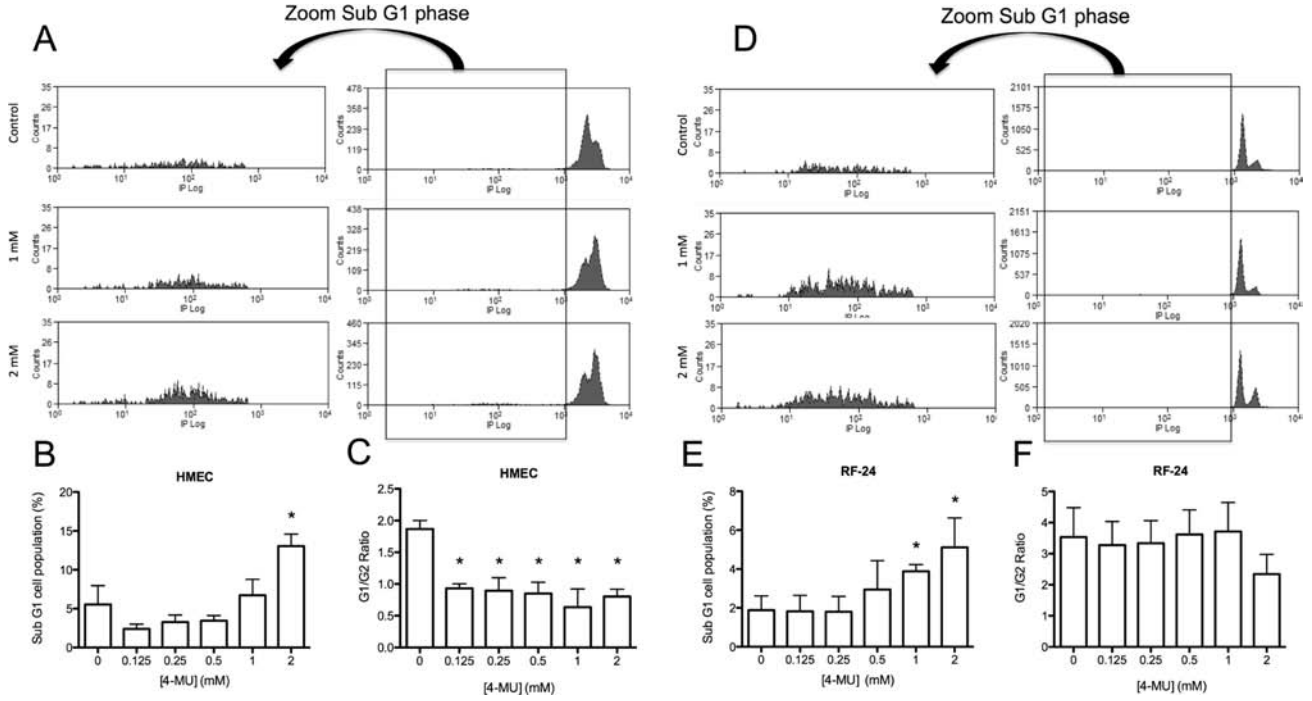

Figure 3. Effects of 4-MU on endothelial cell cycle: (A) representative histograms of human microvascular endothelial cells (HMEC) cell cycle analysis by flow cytometry at different 4-MU concentrations, with zooms of the region corresponding to the sub G1 phase; (B) percentages of HMEC in the sub G1 subpopulation at different 4-MU concentrations; (C) G1/G2 ratio values for HMEC at different 4-MU concentrations; (D) representative histograms of RF-24 cell cycle analysis by flow cytometry at different 4-MU concentrations, with zooms of the region corresponding to the sub G1 phase; (E) percentages of RF-24 cells in the sub G1 subpopulation at different 4-MU concentrations; (F) G1/G2 ratio values for RF-24 cells at different 4-MU concentrations. Experiments were carried out as described under Materials and Methods. Data represent the mean ± SD of three independent experiments. Symbols indicate significant differences between control-untreated and treated cells (∗, $p < 0.05$).

vessels were observed with a binocular lens with filters for fluorescence, and photographs were taken. Procedures carried out with animals complied with the guidelines for animal care of our university and with international guidelines.

Statistical Analysis. For all of the assays, at least three independent experiments were carried out. Results are expressed as the mean ± SD. Statistical significance was determined by using Student's paired sample test and one-way ANOVA. Values of $p < 0.05$ were considered to be significant.

RESULTS

4-MU Inhibits Endothelial Cell Growth. During angiogenesis, local proliferation of endothelial cells does occur. Figure 2 shows that 4-MU inhibits the proliferation of actively growing HMEC and RF-24 endothelial cells in a dose–response manner. From the shown curves, the half-maximal inhibitory concentration (IC_{50}) values could be calculated as 0.65 ± 0.04 and 0.37 ± 0.03 mM for HMEC and RF-24 cells, respectively.

4-MU Alters Endothelial Cell Cycle Distribution and Slightly Induces Endothelial Cell Apoptosis. Figure 3 shows that 4-MU treatment in the millimolar range for 24 h induced changes in the cell cycle distribution, with a slight but significant increase in the number of apoptotic cells (sub G1 subpopulation). On the other hand, even submillimolar concentrations of 4-MU decreased the G1/G2-M ratio in HMEC, whereas 4-MU had no effect on RF-24 G1/G2-M ratio.

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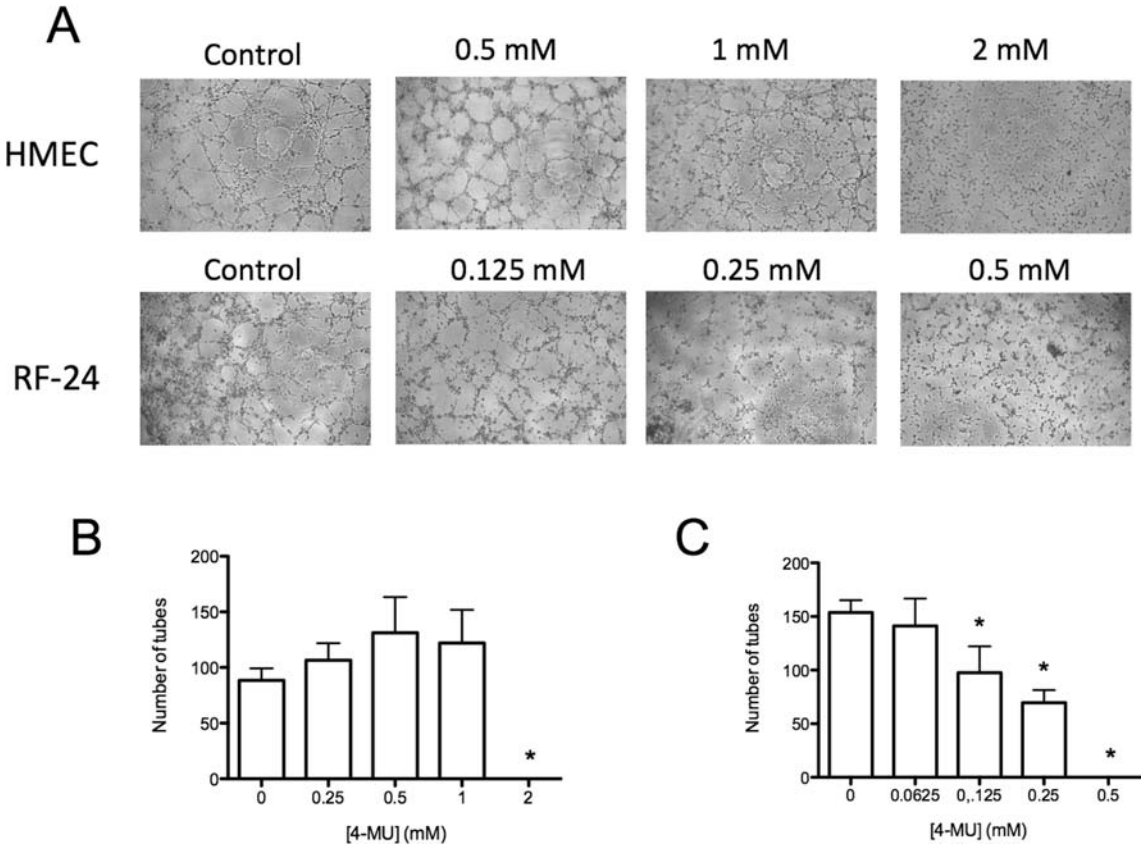

Figure 4. Effects of 4-MU on the formation of "tubule-like" structures of endothelial cells on Matrigel: (A) representative photographs of control (untreated) and 4-MU-treated endothelial cells on Matrigel after 5 h of treatment; (B) quantitative analysis of data for the full range of tested 4-MU concentrations in HMEC; (C) quantitative analysis of data for the full range of tested 4-MU concentrations in RF-24 cells. Experiments were carried out as described under Materials and Methods. Data represent the mean ± SD of three independent experiments. Symbols indicate significant differences between control-untreated and treated cells (∗, $p < 0.01$).

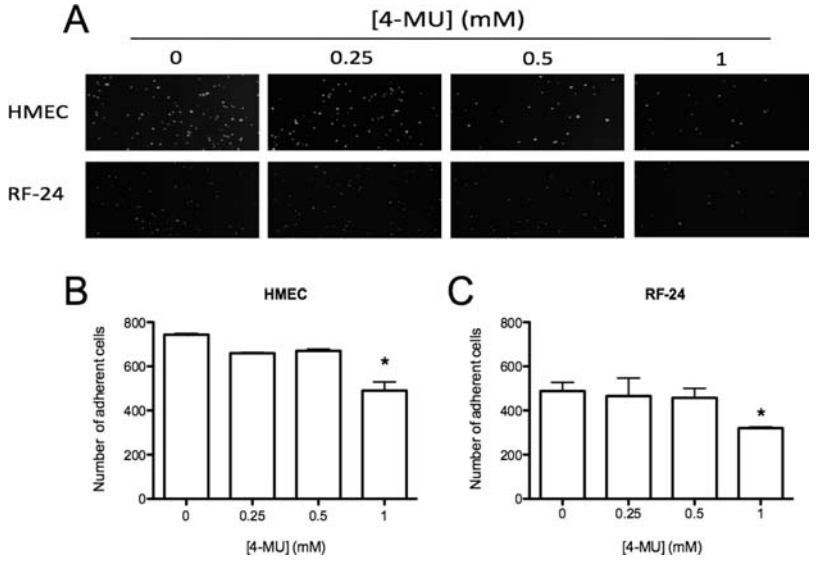

Figure 5. Effects of 4-MU on the adhesion of endothelial cells on gelatin-coated plates: (A) representative photographs of control (untreated) and 4-MU-treated endothelial cells; (B) quantitative analysis of data for the full range of tested 4-MU concentrations in HMEC; (C) quantitative analysis of data for the full range of tested 4-MU concentrations in RF-24 cells. Experiments were carried out as described under Materials and Methods. Data represent the mean ± SD of three independent experiments. Symbols indicate significant differences between control-untreated and treated cells (∗, $p < 0.01$).

4-MU Inhibits the Capillary Tube Formation by Endothelial Cells on Matrigel. At the end of the angiogenic process, there is a rearrangement of endothelial cells in a three-dimensional network of newly formed tubes. The effects of test compounds on this key step of angiogenesis can be studied by using the in vitro assay of formation of tubular-like structures

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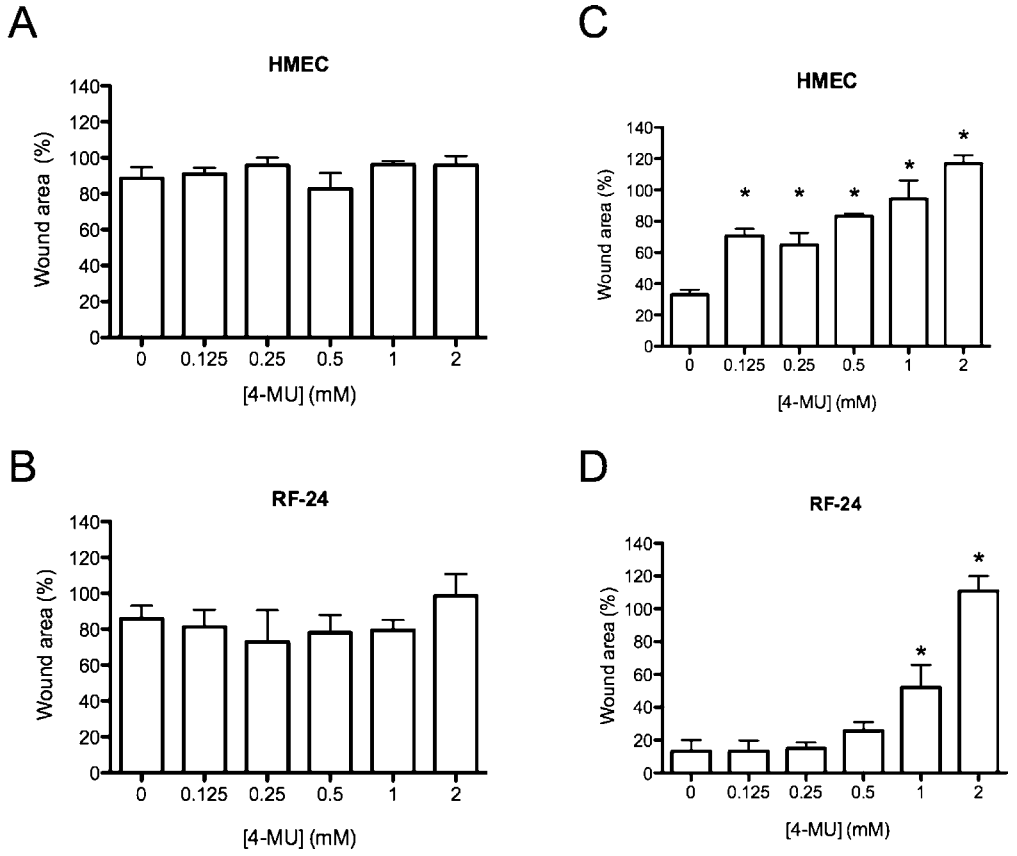

Figure 6. Effects of 4-MU on endothelial cells migration: (A) quantitative analysis of data for the full range of tested 4-MU concentrations in HMEC after 7 h of treatment; (B) quantitative analysis of data for the full range of tested 4-MU concentrations in RF-24 cells after 7 h of treatment; (C) quantitative analysis of data for the full range of tested 4-MU concentrations in HMEC after 24 h of treatment; (D) quantitative analysis of data for the full range of tested 4-MU concentrations in RF-24 cells after 24 h of treatment. Experiments were carried out as described under Materials and Methods. Data represent the mean ± SD of three independent experiments. Symbols indicate significant differences between control-untreated and treated cells (∗, $p < 0.01$).

on Matrigel. Figure 4 shows that 4-MU was able to inhibit endothelial cell alignment and cord formation on Matrigel after 5 h of treatment. The minimal concentrations able to induce complete inhibition in this assay were 2 and 0.5 mM for HMEC and RF-24 endothelial cells, respectively. These data show that the inhibitory effect is much stronger for RF-24 than for HMEC. The required concentrations of 4-MU to induce these inhibitory effects did not affect endothelial cell viability after 5 h (results not shown).

4-MU Decreases the Adhesiveness of Endothelial Cells. During angiogenesis, proliferative endothelial cells must migrate into its surrounding space by a double action of rearrangement of extracellular matrix (ECM) and adhesive contacts with components of this ECM. To test whether 4-MU was able to affect endothelial cell adhesion to ECM components, we carried out an adhesion assay on gelatin after treatments for 24 h. Figure 5 clearly shows that 4-MU partially inhibited the adhesion of endothelial cells to gelatin. Statistically significant differences with respect to control, untreated cells were observed at 1 mM 4-MU for both HMEC and RF-24 endothelial cells.

4-MU Has No Short-Term Effect on the Migratory Capability of Endothelial Cells. Figure 6 shows that after 7 h of treatment, there was no relevant effect of 4-MU on endothelial cell migration in a scratch wound assay. In contrast, there was a clear dose−response inhibitory effect of 4-MU in the same assay after 24 h of treatment. However, at this time of treatment, additional effects on adhesion superpose with the effects on migration, rendering this mixed, complex long-term effect.

4-MU Alters the Expression Levels of Endothelial Cell MMP-2. As mentioned, angiogenesis involves a great remodeling of ECM. After a proangiogenic stimulus, endothelial cells that have acquired the so-called angiogenic phenotype become able to degrade the basal membrane surrounding the vases. In this step, matrix metalloproteinase 2 (MMP-2) plays a key role. Gelatin zymographies of conditioned media and cell extracts of both untreated and treated endothelial cells clearly show that the levels of MMP-2 in both conditioned media and cell extract samples from 4-MU-treated HMEC cells were higher than those of untreated cells (Figure 7A,C). In contrast, 4-MU treatment induced a strong decrease in the expression levels of MMP-2 from both RF-24 cell conditioned media and cell extract (Figure 7B,D).

4-MU Inhibits in Vivo Angiogenesis in the Chick Chorioallantoic Membrane (CAM) Assay. Given the positive and negative results obtained in different in vitro assays, it seemed advisable to test the global potential of 4-MU as a modulator of angiogenesis by using some in vivo angiogenesis assays. In this work, we made use of two in vivo angiogenesis assays, and the results are presented in this and the following section.

Figure 8 shows a typical result obtained in the CAM assay. It can be observed that in controls, vessels formed a dense and

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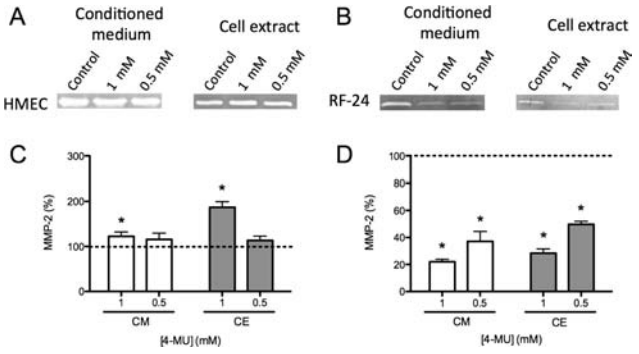
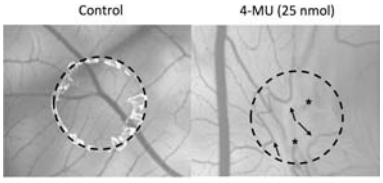


Figure 7. Effects of 4-MU on the levels of MMP-2 activity in endothelial cell conditioned media and cell extracts: (A) representative results of a gelatinolytic assay showing the levels of MMP-2 activity in control (untreated) and 4-MU-treated HMEC conditioned media and cell extracts; (B) representative results of a gelatinolytic assay showing the levels of MMP-2 activity in control (untreated) and 4-MU-treated RF-24 cells conditioned media and cell extracts; (C) quantitative analysis of data for HMEC, taking the control values as 100% (dashed line); (D) quantitative analysis of data for RF-24 cells, taking the control values as 100% (dashed line). Experiments were carried out as described under Materials and Methods. Data represent the mean ± SD for three independent experiments. Symbols indicate significant differences between control-untreated and treated cells (∗, $p < 0.05$).



Dose (nmol/CAM)	Positive/total (%)
0	0/4 (0)
10	0/6 (0)
25	4/7 (57)
50	4/4 (100)
100	5/5 (100)
200	3/3 (100)

Figure 8. Effects of 4-MU on in vivo angiogenesis, as determined by the chorioallantoic membrane (CAM) assay. Areas covered by disks are delimited by dashed circles. Arrows point to rebound of vessels outward from the treated area. Asterisks indicate disrupted vessels. The table summarizes the results obtained in this assay. Experiments were carried out as described under Materials and Methods.

spatially oriented branching network of vascular structures with progressively smaller diameters as they branch. The inhibitory effects of 4-MU can be observed as a general decrease of ingrowth of new vessels in the area covered by the methylcellulose disks containing the compound, as well as a centrifugal growth of the vessels in peripheral positions relative to that of the disk (arrows in Figure 8). The table in Figure 8 shows that this is another dose-dependent inhibitory effect, with 100% in all of the processed samples for treatments with ≥50 nmol per CAM and >50% of positive inhibitory effect for treatments with 25 nmol per CAM.

4-MU Inhibits in Vivo Angiogenesis in a Zebrafish Embryo Assay. The second in vivo angiogenesis assay used in the present study makes use of embryos from a transgenic (TG(fli1:EGFP)y1) zebrafish line. These embryos carry a 15 kb promoter of the transcription factor friend leukemia virus integration-1 (fli-1), which drives the GFP expression in the endothelium.[30] Therefore, fluorescence-labeled embryos can be observed and analyzed as described under Materials and Methods. During development of the zebrafish, intersegmental vessels sprout and grow upward from the aorta, and then the tips join by anastomosis to form a dorsal vein. Figure 9 shows

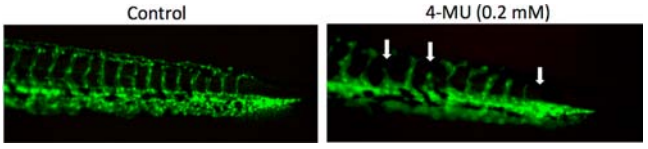


Figure 9. Effects of 4-MU on in vivo angiogenesis, as determined by the assay with the zebrafish model. The arrows points to narrower and disrupted intersegmental vessels in 4-MU-treated zebrafish embryos. Experiments were carried out as described under Materials and Methods. Results were consistently reproduced, and epresentative figures are shown.

that a 24 h treatment with 0.2 mM 4-MU inhibited the proper growth of some of these intersegmental vessels. This can be taken as a clear sign of the antiangiogenic effect of the compound.

DISCUSSION

In this work, we show that 4-MU behaves as an antiangiogenic compound by inhibiting key steps of the process. 4-MU (7-hydroxy-4-methylcoumarin) is a molecule of vegetal origin that has been safely used in humans as a choleretic and spasmolytic compound,[31] as well as in clinical trials for patients with chronic hepatitis B and C (ClinicalTrials.gov identifier NCT00225537). Nonetheless, there is still a lack of convincing clinical or experimental evidence to demonstrate 4-MU is safe for long-term use in therapy. It has been shown that administration of 4-MU leads to a rapid reduction in the levels of hyaluronan.[23] This seems to be the final effect of two complementary actions: on the one hand, 4-MU down-regulates the expression of both hyaluronan synthases 2 and 3,[18] and, on the other hand, 4-MU depletes UDP-glucuronic acid, a precursor for the synthesis of hyaluronan, by activation of UDP-glucoronyl transferases.[21] The mechanisms of action of 4-MU are complex and far from being completely clarified. Its bioactivities have been found in vitro when 4-MU was used in the high micromolar to millimolar range of concentrations.[26−28,32] The effect of 4-MU on hyaluronan availability has been associated with its reported antiproliferant, proapoptotic, and antimigratory effects on several types of cells.[22−28] On the other hand, 4-MU has also antitumor and antimetastasic activity. In a recent report on the antitumor activity of 4-MU in prostate cancer cells,[27] a decreased density of microvessels in a tumor xenograft model is shown, suggesting a potential effect of 4-MU on angiogenesis. However, a thorough study of 4-MU as a modulator of angiogenesis had not been carried out so far. The present work offers the first experimental evidence (both from in vitro and in vivo assays) showing that 4-MU is a new inhibitor of key steps of angiogenesis.

Previous basic research has provided detailed knowledge of the sequence of events required for angiogenesis. This fact and the availability of cultured endothelial cell models have allowed for the development and extensive use of in vitro assays to evaluate the effect of new potential angiogenesis modulators on the different steps of the process. Any compound able to inhibit one or several of these key events can be preselected as a candidate for the inhibition of angiogenesis in vitro.[7]

This work shows that 4-MU inhibits the growth of both immortalized RF-24 and HMEC in a dose-dependent manner.

The IC_{50} values for this activity are in the high micromolar range, as previously observed for other types of cells, including tumor and smooth muscle cells.[24−27,33,34] These growth inhibitory effects of 4-MU on endothelial cells could be due to, at least in part, an induction of apoptosis, because 4-MU has been previously shown to behave as a strong inducer of apoptosis in other cell types when used in the high micromolar to millimolar range of concentrations. The flow cytometric cell cycle distribution analysis carried out in the present work indicates that, in fact, 4-MU is able to induce endothelial cell apoptosis, as revealed by the slight but significant increase in the subG1 subpopulation. Furthermore, these flow cytometric experiments also show a decrease in the HMEC G1/M-G2 ratio, suggesting that 4-MU might induce stagnation of HMEC at the G2 or at the metaphase checkpoints. It is noteworthy that this effect could not be observed in RF-24 cells. The actual involvement of 4-MU on endothelial cell cycle deserves to be further analyzed in the future.

A key in vitro assay to test the antiangiogenic potential of a compound is that of formation of tubular-like structures on Matrigel. Our results in this assay clearly show the inhibitory effect of 4-MU. It is noteworthy that this inhibitory effect is much stronger for RF-24 than for HMEC, because total inhibition of tubular-like structure is observed from 0.5 mM 4-MU on in the case of RF-24 cells, whereas total inhibition is observed only at 2 mM 4-MU in the case of microvascular endothelial cells.

In the present work, we show for the first time an inhibitory effect of 4-MU on the capability of endothelial cells to adhere to ECM components, a key feature required for proper migration of proliferative endothelial cells in activated neovascularization. This observation could be related with the previously shown inhibitory effect of 4-MU on cell−cell adhesion by decreasing *N*-cadherin expression.[32] On the other hand, it has been previously demonstrated that 4-MU also decreases monocyte-smooth cell adhesions.[24]

4-MU has been previously shown to have a clear inhibitory effect on the motility of smooth muscle and tumor cells.[24,28] In the present work, results obtained with the scratch wound assay show no relevant short-term effect induced by 7 h treatments in the presence of 4-MU. In contrast, after 24 h, there was an apparent strong inhibitory effect of endothelial cell migration. This long incubation time was used by others in previous studies on the antimigratory potential of 4-MU.[28] However, in our hands, this long-term incubation was accompanied by an accumulation of detached cells from the dish. This indicates that the final long-term effects observed in this assay could be a combination of the specific effects of 4-MU on adhesion and migration, making difficult the discussion of these data. The effects of 4-MU on the migratory potential of endothelial cells deserve to be further analyzed in the future.

The effect of 4-MU on MMPs is complex, and apparently contradictory results have been published. In cultured human skin fibroblasts, 4-MU induces the expression of membrane type 1 MMP, resulting in an activation of MMP-2.[35] In contrast, the same group has also shown that 4-MU suppresses the expression of MMP-9 (another metalloproteinase with gelatinase activity) in cultured cancer cells.[36] More recently, it has been reported that 4-MU does not affect MMP-2 activity in aortic smooth muscle cells.[24] In the present work, gelatin zymography assays clearly show that 4-MU treatment induces increased levels of MMP-2 in both conditioned media and cell extracts of HMEC, whereas it decreases MMP-2 levels in both conditioned media and cell extracts of RF-24 cells. This remarkable difference in the responses of MMP-2 to 4-MU treatment in microvascular and umbilical vein endothelial cells should be further studied in the future.

Altogether, our results in the in vitro assays point to some specificity of the antiangiogenic effects of 4-MU, with clear inhibitory effects on endothelial cell proliferation, "differentiation", and adhesion. At the same time, 4-MU has no relevant short-term effect on endothelial cell migration. Finally, 4-MU shows contrasting effects on the levels of MMP-2 in HMEC and RF-24 cells. Despite this last apparently proangiogenic effect in the case of HMEC, the rest of the effects on other essential steps of angiogenesis agree well with the observed inhibitory effects of 4-MU in two in vivo angiogenesis assays, namely, the chick CAM and the live fluorescent zebrafish embryo neovascularization assays. On the other hand, the global antiangiogenic potential of 4-MU agrees well with its described effect as a hyaluronic acid biosynthesis specific inhibitor.[18−21] Hyaluronan associations with collagens are known to play an important role in the initiation and maintenance of angiogenesis.[37] Furthermore, hyaluronic acid fragments do enhance angiogenesis.[38] On the other hand, hyaluronan synthase-2, which stimulates cell proliferation and angiogenesis,[39] has been shown to reduce its synthetic activity in the presence of 4-MU.[18]

A remarkable number of plant-derived compounds with a wide diversity of chemical structures have been reported to inhibit angiogenesis.[12,13,40−45] The results of the present work add 4-MU to this growing list, as a compound able to inhibit both in vitro and in vivo angiogenesis. Interestingly, its specific effects on multiple steps of the angiogenic process could open new venues relating this compound with potential pharmacological and/or chemopreventive strategies against angiogenesis-dependent pathologies, including cancer.[1,2,8] Additional experimental efforts will be required to elucidate the molecular mechanisms underlying the antiangiogenic activity of 4-MU. On the other hand, hyaluronic acid is a critical component distributed widely throughout connective, epithelial, and neural tissues under normal physiological condition. Thus, the long-term use of hyaluronic acid biosynthesis inhibitors, such as 4-MU, could have potential risk to cause systemic damages. Therefore, rigorous pharmacokinetics, pharmacodynamics, and risk assessment studies would be required before any potential use of 4-MU as an antiangiogenic compound could enter the clinics.

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Notes

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■ ABBREVIATIONS USED

4-MU, 4-methylumbelliferone; CAM, chorioallantoic membrane; HMEC, human microvascular endothelial cell; MMP-2, matrix metalloproteinase-2

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