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A novel 2,3-diphenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one derivative inhibits endothelial cell dysfunction and smooth muscle cell proliferation/activation

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ABSTRACT

Hyper-proliferation and migration of vascular smooth muscle cells and endothelial cell dysfunction are central events in the development of neo-intimal lesions.

Pursuing our interest in the synthesis of bioisosters of flavonoids, we studied in depth a novel synthetic 2,3-diphenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one derivative, examining its effects in vitro on induced-cell proliferation and activation in human aortic smooth muscle cells (HAoSMCs) and in human umbilical vein endothelial cells (HUVECs).

Compared with two well known flavonoids, apigenin and quercetin, the novel compound, 2-(3,4-dimethoxyphenyl)-3-phenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one, **3**, was not toxic for HUVECs, even at high concentrations and for long incubation times, while the two flavonoids were not tolerated, even at concentrations as low as 10 μmol/L. Compound **3** inhibited selectively, and in a concentration-dependent manner, the proliferation of HAoSMCs but not that of HUVECs. In HUVECs, it inhibited the cytokine-induced vascular cell adhesion molecule-1 expression, but not the cyclooxygenase-2 (COX-2) expression. Instead, in HAoSMC, it inhibited the induction of COX-2 expression and the relative release of prostaglandin E_2. In addition, it inhibited the transcription of the matrix metalloproteinase-9 and its activity.

Thanks to its multiple and tissue-specific function, 2-(3,4-dimethoxyphenyl)-3-phenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one might replace or assist the action of current drugs eluted by coronary stents, in order to promote a functional repair of damaged wall.

1. Introduction

Percutaneous coronary intervention remains an effective therapy for the revascularization of occluded arteries [1,2]. However, at the site of endovascular intervention, we observe endothelial denudation and dysfunction, characterized by an increase in inflammatory cytokines, enhanced expression of adhesion proteins and cyclooxygenase-2 (COX-2) [3,4]. Injured and inflamed vessel wall attracts platelets and leukocytes that release growth factors and cytokines, promoting also activation of vascular smooth muscle cells, which migrate to de-endothelialized vessel surface and proliferate. All this leads to neo-intimal tissue formation which, in turn, may lead to vessel restenosis [4–6]. Therefore, injured vessel wall should be profitably treated with agents possessing vascular protective properties, improving vascular healing, protecting the endothelial layer, and down-regulating smooth muscle cell proliferation and migration [7].

The development of neointima can be critically induced by cytokines and growth factors such as tumor necrosis factor (TNF-α) and platelet derived growth factor-BB (PDGF-BB), as well established in arterial injury models [8,9]. Therefore, the inhibition of TNF-α-induced endothelial dysfunction and PDGF-BB-stimulated vascular smooth cell proliferation and phenotypic modulation,

Abbreviation: HAoSMCs, human aortic smooth muscle cells; HUVECs, human umbilical vein endothelial cells; COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor-α; PDGF-BB, platelet derived growth factor-BB; ECGF, endothelial cell growth factor; VCAM-1, vascular cell adhesion molecule-1; MMP-9, matrix metalloproteinase-9; PGE_2, prostaglandin E_2; PGI_2, prostacyclin; PMA, phorbol 12-myristate 13-acetate.

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may represent an important point of therapeutic intervention in restenosis after angioplasty.

However, the currently available anti-proliferative drugs eluted from coronary stents, while inhibiting neo-intimal hyperplasia, impair the antithrombotic functions of endothelial cells, hindering the re-endothelialization and thereby increasing the risk of stent thrombosis [2,10,11]. Accordingly, novel and more effective therapeutic agents are highly welcome.

Flavonoids, a family of natural polyphenolic compounds, show many biological and pharmacological effects, resulting from their antioxidant, anti-inflammatory, and anti-angiogenetic activities [12]. Although they are characterized by low bioavailability [13,14], a huge amount of experimental data, acquired through both in vitro assays and in animal models, clearly demonstrate their ability to modulate key cellular and molecular mechanisms, related to cardiovascular diseases and some types of cancer [8,15–18]. Accordingly, they represent an intriguing source of inspiration for medicinal chemists, who are involved in a continuous production of structurally related synthetic analogs, developed with the aim of achieving clinically effective compounds endowed with suitable pharmacokinetic properties.

Pursuing our interest in the synthesis of bioisosters of flavonoids, we moved from our previously developed anti-oxidant derivatives, characterized by a 2-phenyl-4*H*-pyrido[1,2-*a*]pyrimidine core [19] and, through the insertion of an additional phenyl ring in the position 3 of the heterocyclic scaffold, we obtained a novel class of 2,3-diphenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-ones (DPPPs, Chart 1). The synthesized compounds, designed as anti-oxidant products provided with anti-inflammatory activity, turned out to be effective and viable agents exploitable in the management of vascular dysfunctions [20].

Here we describe the functional evaluation of the main representative of this novel class of compounds, 2-(3,4-dimethoxy phenyl)-3-phenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one, **3**, whose efficacy as a novel drug candidate for the treatment of vessel walls subjected to endovascular intervention has been studied in depth, through in vitro assays on both endothelial cells and smooth muscle cells.

2. Experimental protocol

2.1. Materials

All reagents were purchased from Sigma–Aldrich (St. Louis, MO) except where specified. Cell Proliferation Reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulfonate), and Cell Proliferation ELISA, BrdU (colorimetric) were obtained from Roche Diagnostics (Mannheim, Germany). The goat anti-COX-2 polyclonal antibody was purchased from Tema Ricerca Srl (Bologna, Italy). PGI_2 (as 6-keto-PGF1*a*) and prostaglandin E_2 (PGE_2) (enzyme immunoassay, EIA) kits were purchased from Cayman Chemical Company (Ann Arbor, MI, USA).

Stock solutions of apigenin, quercetin and test compounds were dissolved in sterile dimethyl sulfoxide (DMSO) and stored at −80 °C at the maximum solubility of 50 mM. Since the final concentration of DMSO in the culture medium never exceeded 0.1% (vol/vol), DMSO (0.1%) alone served as the control. At this concentration, DMSO alone, used as the control, did not show any effect on cell viability, cell proliferation, or related molecular mechanisms (data not shown).

2.2. Chemical synthesis of test compounds

2,3-Diphenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-ones (Chart 1), were synthesized by S.S., B.D., and C.L.M. (Department of Pharmacy) as reported elsewhere [20]. The target compound, 2-(3,4-dimethoxyphenyl)-3-phenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one **3**, in particular, was obtained from the key intermediate 2-(3,4-dimethoxyphenyl)-4*H*-pyrido[1,2-*a*]pyrimidin-4-one [19]. A suspension of the key intermediate (1.00 mmol) and *N*-bromosuccinimide (1.00 mmol) in chloroform was refluxed under stirring until the disappearance of the starting material (TLC analysis). The solvent was then removed in vacuo and the crude product, 3-bromo-2-(3,4-dimethoxyphenyl)-4*H*-pyrido[1,2-*a*]pyrimidin-4-one, was purified by crystallization from EtOH. The pure bromo-derivative (1.00 mmol) was then added to a suspension of bis(triphenyl phosphine)palladium(II) dichloride (0.20 mmol) in EtOH/toluene, followed by phenylboronic acid (1.50 mmol) and an aqueous solution of Na_2CO_3 (2 M, 3.0 mL). The resulting reaction mixture was refluxed under stirring until the disappearance of the starting material (TLC analysis). After being cooled to room temperature, the mixture was evaporated to dryness under reduced pressure and the residue was purified by crystallization from MeOH, to afford the target compound, **3**, as a white solid. Yield: 90%. P. f.: 182–184 °C. ^{1}H NMR, δ, ppm, Hz: 8.96 (d, 1H, $J = 7.08$), 7.95 (t, 1H, $J = 8.79$), 7.73 (d, 1H, $J = 8.79$), 7.34–7.10 (m, 7H), 6.88–6.75 (m, 2H), 3.72 (s, 3H), 3.36 (s, 3H).

2.3. Cell cultures and treatments

Human umbilical vein endothelial cells (HUVECs) were isolated, characterized and maintained as described [21]. Cells were obtained from discarded umbilical vein and treated anonymously conforming to the principles outlined in the Declaration of Helsinki. Cells were used up to the fifth passage from primary culture. Human aortic smooth muscle cells (HAoSMCs) were obtained from the American Type Culture Collection (CRL 1999; ATCC, USA), cultured with HAM'S F-12K medium supplemented with 10% fetal bovine serum, as described previously [22] and used between passages 20–30.

If not otherwise indicated, the experiments were performed when the cells reached the confluence, and cells were pre-treated for 1 h with 1, 10 and 25 μM of compound, or vehicle alone, before each challenge. The stimulation occurred with phorbol 12-myristate 13-acetate (PMA) (10 nM), PDGF-BB (20 ng/mL), endothelial cell growth factor (ECGF) (20 ng/mL) or TNF-α (10 ng/mL).

2.4. Cell toxicity assay

Cellular toxicity by quercetin, apigenin and the novel synthesized compounds, 2,3-diphenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-ones, was checked for concentrations up to 50 μM through phase contrast microscopy of cell morphology and WST-1 assay.

The quantitative cell viability was assessed with the compound WST-1 (Roche Diagnostics) following the manufacturer's protocol. This assay reflects the activity of mitochondrial dehydrogenase present in living cells. During the assay, the yellow tetrazolium salt WST-1 is reduced to a highly colored formazan dye by mitochondrial enzymes in cells. Because these mitochondrial enzymes are inactivated shortly after cell death, the orange colored formazan dye only appears in viable cells. Cell grown on 96-well plates were treated with quercetin, apigenin or 2,3-diphenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-ones for 24 h or 72 h, and subsequently treated with premix solution (10 μL) for further 2 h. Afterward, the absorbance at 450 nm was read using a microplate reader.

2.5. Cell proliferation assay

A colorimetric immunoassay (Roche Diagnostic) based on the measurement of BrdU incorporation during DNA synthesis was

used. Prior to stimulation, cells, at a confluence of about 70%, were synchronized by overnight serum starvation. Then, cells pre-treated with **3** were stimulated with PDGF-BB or ECGF for 48 h. Subsequently cells were labeled with BrdU, fixed, and stained according to the manufacturer's instructions. Finally, the absorbance at 450 nm was read using a microplate reader.

2.6. Detection of cell surface VCAM-1 expression

HUVECs, grown in 96-well plates, were pre-treated with the novel synthesized compounds, 2,3-diphenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-ones, and stimulated with TNF-α overnight. The VCAM-1 expression was measured by a cell surface enzyme immunoassay, as previously described by us [23].

2.7. COX-2 protein expression assay

Cells were grown in 96-well plates and exposed to **3** before PMA, PDGF-BB or TNF-α overnight stimulation. A modified EIA procedure was used to measure the cellular expression of COX-2. Briefly, cell monolayer was washed once with cold phosphate-buffered saline before being fixed and permeabilized on ice with cold acetone for 2 min. EIA were then carried out by incubating the monolayer first with saturating concentrations (1 μg/mL) of goat anti-COX-2 antibody (Tema Ricerca Srl), then with HRP-conjugated rabbit anti-goat IgG antibody. The plates were washed three times between each incubation step and at each time the monolayer integrity was monitored by microscopy. The peroxidase substrate, 3,3′,5,5′-tetramethylbenzidine was then added to the micro-well. After 30 min, the reaction was stopped by adding 0.18 M sulfuric acid (50 μL) and COX-2 cellular content was finally quantified spectrophotometrically, reading the absorbance at 450 nm.

2.8. Total RNA extraction and reverse-transcription PCR (RT-PCR)

Cells were cultured in 21 cm^2 dishes, exposed to **3** and stimulated with PMA, PDGF-BB or TNF-α overnight (for COX-2 and matrix metalloproteinase-9 [MMP-9] mRNAs) or for 4 h (for VCAM-1 mRNA). Total RNA was isolated using QIAzol lysis reagent (QIAGEN, Hilden, Germany) and quantified spectophotometrically at 260 nm. The ratio of O.D. values at 260 nm and 280 nm provided an estimate of RNA purity. For each sample, the RNA integrity was evaluated by electrophoresis in a 1.5% agarose gel.

Next, RNA (1 μg) was reverse-transcribed to cDNA by iScript cDNA Synthesis Kit (Biorad, Hercules, CA, USA) in 20 μL of total reaction volume, according to the manufacturer's instructions in a GeneAmp PCR System 9700 thermal cycler (Perkin Elmer, Lockport Place, Lorton).

The gene expression of VCAM-1, MMP-9 and COX-2 was performed using specific primers and conditions (Table 1) as previously described [21,24,25]. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified as an internal control (Table 1) in the same reaction of the target genes. All PCR reactions were performed in a 50 μL total volume, containing *Taq* polymerase (Qiagen, Germany) (2.5 U), deoxyribonucleotide triphosphate (dNTP) (0.2 mM), $MgCl_2$ (1.5 mM) and forward and reverse primers (1 μM each). The PCR products were separated by electrophoresis on an agarose gel (2%) in Tris/acetate/EDTA buffer and visualized at 260 nm after staining with ethidium bromide. After gel image acquisition by a digital camera (Kodak DC290 Digital camera System™; Eastman Kodak, Rochester, NY, USA), band intensity of PCR DNA was quantified using densitometric analysis by Scion image™. The relative mRNA expressions of VCAM-1, MMP-9 and COX-2 were normalized to GAPDH expression.

Table 1
RT-PCR primer pairs.

Genes	Primers (5′-3′)	Length (bp)	Annealing (°C)	Cycles (n)
VCAM-1	F: 5′-ATGACATGCTTGAGCCAGG-3′ R: 5′-GTGTCTCCTTCTTTGACACT-3′	260	55° (60 s)	30
COX-2	F: 5′-TGGGAAGCCTTCTCTAACCTCTCCT-3′ R:5′-CTTTGACTGTGGGAGGATACATCTC-3′	388	52° (30 s)	28
MMP-9	F: 5′-GGCGCTCATGTACCCTATGT-3′ R: 5′-TCAAAGACCGAGTCCAGCTT-3′	468	64° (30 s)	35
GAPDH	F: 5′-GGTCTCCTCTGACTTCAACAGCG-3′ R: 5′-GGTACTTTATTGATGGTACATGAC-3′	354	55° (60 s)	30

2.9. Measurement of PGI_2 and PGE_2 production

Cells grown in 96-well plates were exposed to **3** and stimulated with PMA or TNF-α. The conditioned medium was then collected, centrifuged and concentrations of PGI_2 (as 6-keto-PGF1*a*, the stable metabolite of PGI_2) and PGE_2 were determined by competitive enzyme immunoassays (Cayman Chemical Company), according to manufacturer's instructions. Values obtained were expressed in pg/mL.

2.10. Gelatin zymography

HAoSMCs grown in 21 cm^2 dishes were treated in serum-free medium with PMA in presence/absence of **3** for 24 h. The conditioned media were collected and analyzed for MMP-9 activity by gelatin zymography. The protein content of the samples was measured by the colorimetric method using serum albumin as the standard. Proteins (1 μg) were loaded on SDS-PAGE gel (11%) containing gelatine (0.1%) for electrophoresis at constant voltage (125 V) for approximately 1.5 h in cold room (4 °C). Gels were washed twice with 2.5% Triton X-100 (2.5%) and incubated (24 h at 37 °C) in zymographic buffer [TRIS—HCl (0.5 M) pH 7.5, $CaCl_2$ (50 mM), NaCl (2 M), Brij35 (0.2%)]. Subsequently, gels were stained with Coomassie Brilliant Blue R-250 (0.5%), de-stained with methanol (5%) in acetic acid (10%), and visualized. Clear areas on the Coomassie-stained gel, which indicated the presence of proteolytic activity, were quantified by densitometric scanning. Densitometry of destained areas was quantified with the use of Scion image™.

2.11. Statistical analysis

The results were expressed as mean ± SD or mean ± SEM. Multiple comparisons were performed by one-way ANOVA followed by Bonferroni's post-hoc tests. Values of $P < 0.05$ were considered statistically significant. Data were analyzed with the use of statistical software SPSS 13.0 (SPSS Inc, Chicago, IL, USA).

3. Results

3.1. Effects of 2,3-diphenyl-4H-pyrido[1,2-a]pyrimidin-4-ones, apigenin and quercetin on endothelial cell survival

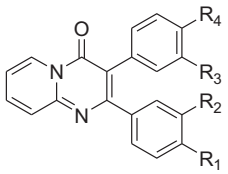
We started our functional study evaluating the toxicity of the novel synthesized 2,3-diphenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-ones on HUVECs, exploiting the WST-1 assay. None of the test compounds displayed any evident toxicity (data not shown). As an example, data obtained with **3** (10, 25 and 50 μM) in HUVECs, with respect to apigenin and quercetin, are reported in Fig. 1A. HUVECs treated with **3**, for 24 h and 72 h, showed no significant difference in the viability when compared to the untreated cells,

whereas apigenin and quercetin resulted in a strong decrease in cell viability, as evaluated by the WST-1 assay. The effects on HUVECs morphology after 72 h of treatment were assessed with phase-contrast microscopy. As shown by photomicrographs (Fig. 1B) **3** did not cause cell detachment while apigenin and quercetin markedly reduced the number of adherent cells (Fig. 1B). Although **3** proved to have no toxic effects on HUVECs up to 50 μM, in the following studies we chose to exploit up to 25 μM of compound, being clinically more compatible. Since flavonoids were toxic at these concentrations and with less exposure time, we cannot compare their efficacy with that of **3**. On the other hand, at lower concentrations they had no effect (data not shown).

3.2. 2,3-Diphenyl-4H-pyrido[1,2-a]pyrimidin-4-ones transcriptionally modulates endothelial VCAM-1 expression

We tested the effect of the novel 2,3-diphenyl-4*H*-pyrido[1,2-*a*] pyrimidin-4-one derivatives on TNF-α induced VCAM expression, used here as a molecular marker of inflammation and endothelial cell dysfunction. As shown in Table 2, all the test compounds

Table 2
Effects of 2,3-diphenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one derivatives, **1**–**8**, on VCAM-1 expression.



N	R_1	R_2	R_3	R_4	% of inhibition[a]
1	H	H	H	H	8.5 ± 3.2
2	OCH_3	H	H	H	8.9 ± 3.3
3	OCH_3	OCH_3	H	H	37 ± 8.5
4	H	H	H	OCH_3	12 ± 4.1
5	H	H	OCH_3	OCH_3	12 ± 3.6
6	OCH_3	H	H	OCH_3	9.6 ± 3.9
7	OCH_3	OCH_3	H	OCH_3	10.3 ± 4.2
8	OCH_3	H	OCH_3	OCH_3	15 ± 3.3

[a] Values ± SEM, determined in HUVECs pre-treated with the test compound (25 μM) for 1 h, stimulated with TNF-α (10 ng/mL) overnight and assayed for cell-surface VCAM-1 expression.

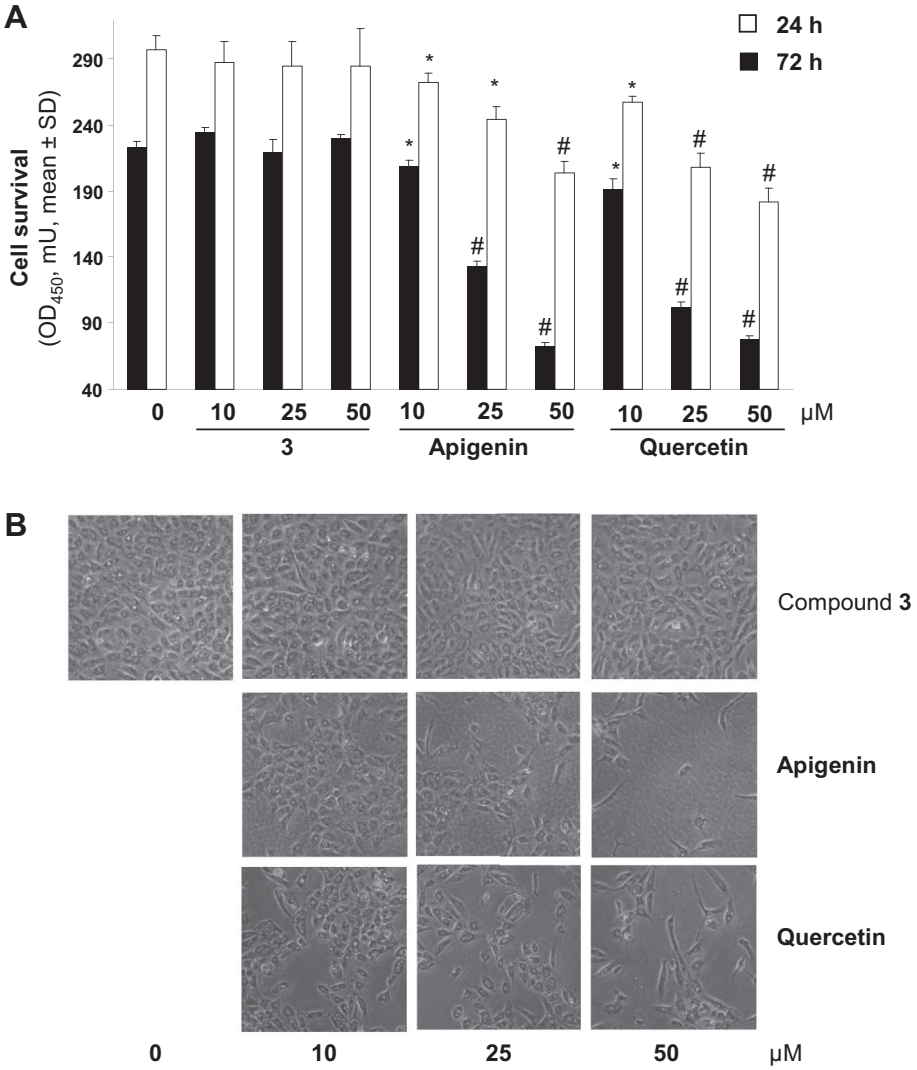


Fig. 1. Effects of **3**, apigenin and quercetin on endothelial cell survival A) HUVECs were pretreated with **3**, apigenin and quercetin for 24 h and 72 h, and a WST-1 assay was performed. Each bar represents the mean of three independent experiments, each performed in $n = 6$ replicates. ANOVA with Bonferroni's post hoc comparison, $^{*}P < 0.01$ and $^{\#}P < 0.001$ vs. control. B) Representative photomicrographs show the effect of the three compounds on HUVEC monolayer after 72 h of treatment.

proved to modulate endothelial VCAM-1 expression, showing percentage inhibition values ranging from 8.5 to 37. Among all the test compounds, **3** exhibited the highest efficacy, demonstrating to inhibit TNF-α induced VCAM-1 expression in a concentration-dependent manner (Fig. 2A). To determine whether the inhibitory effect of **3** on VCAM-1 was due to changes in mRNA levels, a RT-PCR analysis of VCAM-1 gene expression was performed on HUVECs treated with TNF-α in the presence/absence of **3**. VCAM-1 mRNA transcripts were not found expressed in untreated cells while the addition of TNF-α up-regulated VCAM-1 mRNA (Fig. 2B).

Treatment with **3** (10–25 μM) resulted in a significant reduction of VCAM-1 m-RNA expression (Fig. 2B). Conversely, this treatment did not effect the mRNA levels of the constitutively expressed gene GAPDH (Fig. 2B). The effects of **3** on VCAM-1 mRNA levels reflect the results obtained with measurements of cell surface expression of VCAM-1.

3.3. Compound *3* inhibits the smooth muscle cell proliferation but not that of endothelial cells

The development of restenosis is largely due to vascular smooth muscle cell hyper-proliferation [26]. To determine whether **3** has a growth inhibition effect on vascular cells, the BrdU incorporation assay was performed. The test compound inhibited HAoSMCs proliferation, stimulated by the potent growth factor PDGF-BB, in a concentration-dependent manner (Fig. 3A). On the contrary, the proliferation of HUVECs slightly increased after treatment with 1 μM **3**, alone or in the presence of ECGF, when compared to the untreated cells. Treatment with higher concentrations of **3** (10–25 μM) did not show any effects on cell proliferation (Fig. 3B).

3.4. COX-2 expression: compound *3* has differential actions on the two cell types

Since the activation of COX-2 is critically involved in cell proliferation, growth and inflammatory responses [27–29], we explored the effect of our compound on COX-2 protein expression elicited by various stimuli. In HUVECs, COX-2 protein expression was up-regulated by PMA or TNF-α but **3** did not alter the COX-2 expression induced from both compounds (Fig. 4A). Results of COX-2 mRNA, analyzed by RT-PCR, confirmed those of the protein assay (Fig. 4B). According to the irrelevant effect of **3** on COX-2 expression in HUVECs, our compound did not affect the COX-2 activity stimulated by TNF-α, as assessed by measuring the PGI_2/PGE_2 release (Fig. 4C).

Dissimilar results were obtained on HAoSMCs, pre-treated with **3**. COX-2 protein expression in HAoSMCs was highly induced by PDGF-BB (Fig. 5A). Derivative **3** inhibited the PDGF-BB-induced COX-2 expression in a concentration-dependent manner (Fig. 5A). The inhibitory effect occurred at transcriptional levels, as shown by the COX-2 mRNA evaluation (Fig. 5B).

The inhibitory effect of **3** on COX-2 protein and gene expression stimulated by PMA was also investigated, and results obtained showed the same inhibitory pattern of **3** on COX-2 expression induced by PDGF-BB (Fig. 5A and B). In accordance with reduced COX-2 protein expression, **3** suppressed PMA-stimulated COX-2 activity, as assessed by measuring the production of PGE_2 in culture media (Fig. 5C).

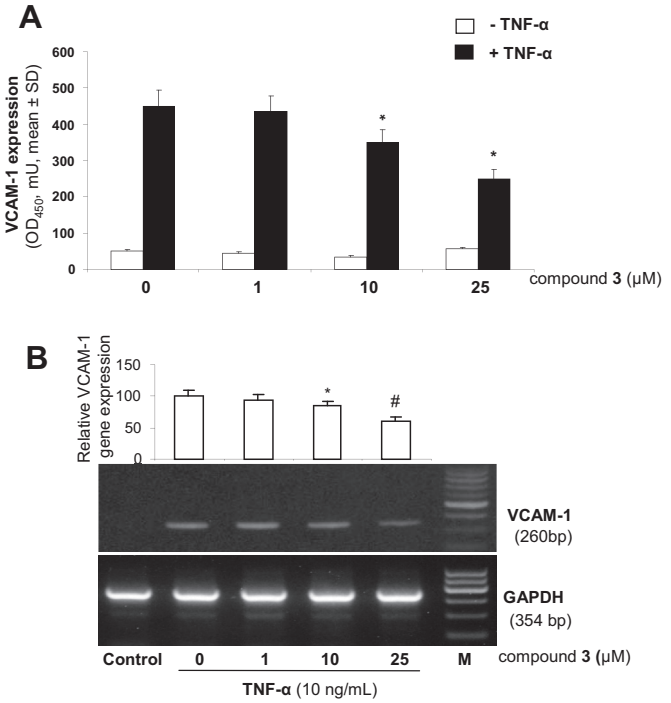


Fig. 2. Effects of **3** on VCAM-1 expression A) HUVECs were pre-treated with **3** for 1 h, and stimulated with TNF-α (10 ng/mL) overnight. Each bar represents the mean of three independent experiments, each performed in $n = 8$ replicates. ANOVA with Bonferroni's post hoc comparison, $^{*}P < 0.001$ vs. TNF-α alone. B) VCAM-1 mRNA, analyzed by RT-PCR, in HUVECs pre-treated with **3** for 1 h and stimulated with TNF-α for 4 h. PCR DNA bands shown are representatives of three determinations. Bar graphs of all values were expressed as the mean ± SEM of three determinations, ANOVA with Bonferroni's post hoc comparison, $^{*}P < 0.05$ and $^{\#}P < 0.01$ vs. TNF-α alone. M = markers.

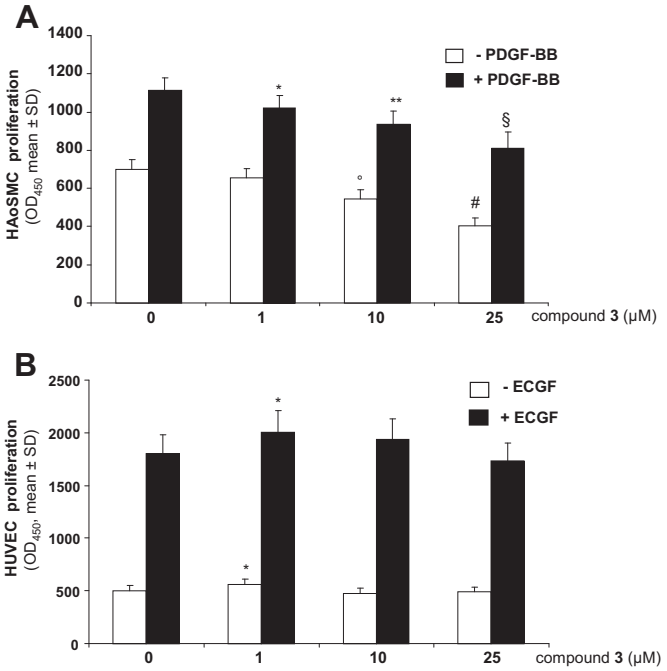


Fig. 3. Effects of **3** on cell proliferation evaluated with the BrdU cell incorporation assay A) Preconfluent HAoSMC pre-treated with **3** for 1 h were grown in the presence/absence of PDGF-BB (20 ng/mL) for 48 h. Each bar represents the mean of three separate experiments each performed in $n = 8$ replicates. ANOVA with Bonferroni's post hoc comparison, $^{*}P < 0.01$, $^{**}P < 0.001$ and $^{\S}P < 0.0001$ vs. PDGF-BB alone, $^{\circ}P < 0.01$ and $^{\#}P < 0.001$ vs. control. B) Preconfluent HUVECs pre-treated with **3** for 1 h, were grown in the presence/absence of ECGF (20 ng/mL) for 48 h. Each bar represents the mean of three separate experiments each performed in $n = 8$ replicates. ANOVA with Bonferroni's post hoc comparison, $^{*}P < 0.01$ vs. ECGF or control.

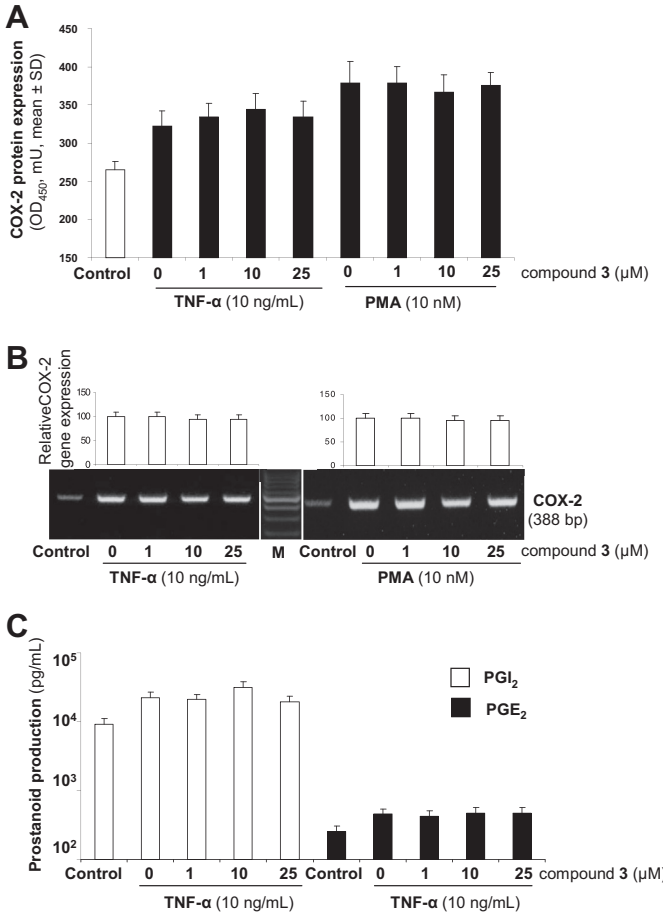


Fig. 4. Effects of **3** on COX-2 expression and prostanoid production in HUVECs A) HUVECs were pre-treated with **3** for 1 h, and stimulated overnight with TNF-α or PMA. The COX-2 protein expression was measured as described in the Methods. Each bar represents the mean of three independent experiments, each performed in $n = 6$ replicates. B) COX-2 m-RNA, analyzed by RT-PCR, in HUVECs pre-treated with **3** for 1 h, and stimulated overnight with TNF-α or PMA. M = markers C). PGI_2 (as 6-keto-PGF1α) and PGE_2 production in HUVECs were measured in cell culture medium after overnight stimulation with TNF-α. Data are means ± SEM of three independent experiments performed in duplicate.

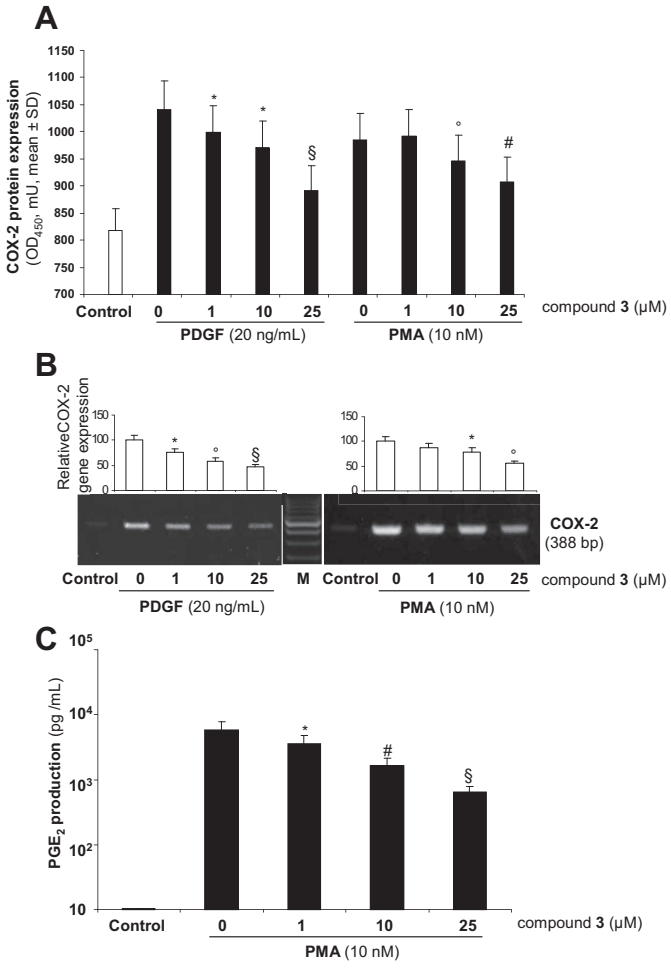


Fig. 5. Effects of **3** on COX-2 expression and PGE_2 production in HAoSMCs A) HAoSMCs were pre-treated with **3** for 1 h, and stimulated overnight with PDGF-BB or PMA. The COX-2 protein expression was measured as described in the Methods. Each bar represents the mean of three independent experiments, each performed in $n = 6$ replicates. ANOVA with Bonferroni's post hoc comparison, $^{*}P < 0.01$, and $^{§}P < 0.001$ vs. PDGF-BB alone; $^{\circ}P < 0.01$ and $^{\#}P < 0.001$ vs. PMA alone. B) COX-2 m-RNA, analyzed by RT-PCR, in HAoSMCs pre-treated with **3** for 1 h and stimulated overnight with PDGF-BB or PMA. ANOVA with Bonferroni's post hoc comparison, $^{*}P < 0.05$, $^{\circ}P < 0.01$ and $^{§}P < 0.001$ vs. PDGF-BB alone; $^{*}P < 0.05$, $^{\circ}P < 0.01$ vs. PMA alone. M = markers. C) PGE_2 production in HAoSMCs was measured in cell culture medium after overnight stimulation with PMA. Data are means ± SEM of three independent experiments performed in duplicate. ANOVA with Bonferroni's post hoc comparison, $^{*}P < 0.01$, $^{\#}P < 0.001$ and $^{§}P < 0.0001$ vs. PMA alone.

3.5. Compound **3** inhibits MMP-9 gene transcription and its enzymatic activity in smooth muscle cells

MMPs may be a potential therapeutic target for the treatment of restenosis or atherosclerosis, as they play a key role in smooth muscle cell migration and neointima formation after vascular injury.

We evaluated the effect of **3** on gene transcription of MMP-9, the main protease involved in the above-mentioned process. HAoSMCs were pre-treated with increasing concentrations of test compound before PMA stimulation, and then mRNA expression of the target proteins was evaluated. As shown in Fig. 6A, the up-regulation of MMP-9 m-RNA induced by PMA was inhibited by **3** at 10 and 25 μM.

To determine whether **3** affected the levels of proteolytic activity of MMP-9, using gelatin zymography, we examined the conditioned medium from HAoSMCs exposed to PMA with or without **3** (10 and 25 μM) for 24 h.

Zymography analysis of HAoSMCs treated with PMA alone revealed the presence of MMP-9 (82 kDa) compared to cells treated with DMSO alone (Fig. 6B). The supernatants harvested from HAoSMCs pre-treated with **3** (25 μM) showed reduced induction of MMP-9 (Fig. 6B).

4. Discussion

In this study, we describe the functional evaluation of a novel heterocyclic compound, 2-(3,4-dimethoxyphenyl)-3-phenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-one, **3**, which emerged as the most effective one among a number of parent analogs developed as drug candidates for the treatment of vascular dysfunction [20]. Since the test compound was structurally inspired by the flavonoid scaffold, we preliminarily evaluated its effects on endothelial cell vitality with respect to two main flavonoids, apigenin and quercetin, whose anti-proliferative and anti-inflammatory properties have been associated with the prevention and therapy of cardiovascular diseases and cancer [30,31].

Surprisingly, in our experimental conditions, the two known flavonoids turned out to be toxic to endothelial cells after 72 h of incubation, even at concentration of 10 μM, while **3** was well-tolerated even at 50 μM and for longer time. Therefore, by

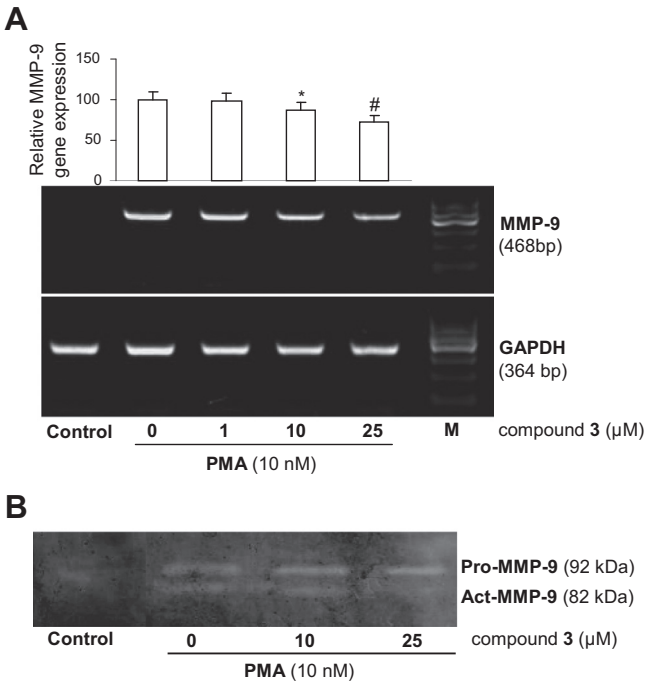


Fig. 6. Effects of **3** on MMP-9 gene expression and activity in HAoSMCs A) MMP-9 mRNA, analyzed by RT-PCR, in HAoSMCs pre-treated with **3** for 1 h and stimulated overnight with PMA. The bands of PCR DNA shown are representatives of three determinations. Bar graphs of all values were expressed as the mean ± SEM from three determinations. ANOVA with Bonferroni's post hoc comparison, $^{*}P < 0.05$ and $^{\#}P < 0.01$ vs. PMA alone. M = markers B) MMP-9 activity was determined by gelatin zymography assay. The bands of enzymatic activity shown are representative of three determinations.

suitably modifying the heterocyclic core of flavonoids we succeeded in obtaining an effective compounds, devoid of any toxic effects and endowed with improved vasoprotective and anti-apoptotic properties. Most cell culture-based studies investigating the inhibitory effects of polyphenols on cell proliferation generally exploit supra-physiological concentrations of specific compounds, thus treating cells with very high apoptotic concentrations (until to 100 μM) [30,31]. On the contrary, our compound inhibited the proliferation of HAoSMCs, responsible for the abnormal neointimal formation, even at low concentrations, without altering endothelial cell vitality, ensuring both tissue homeostasis and the healing of the injured vessel wall.

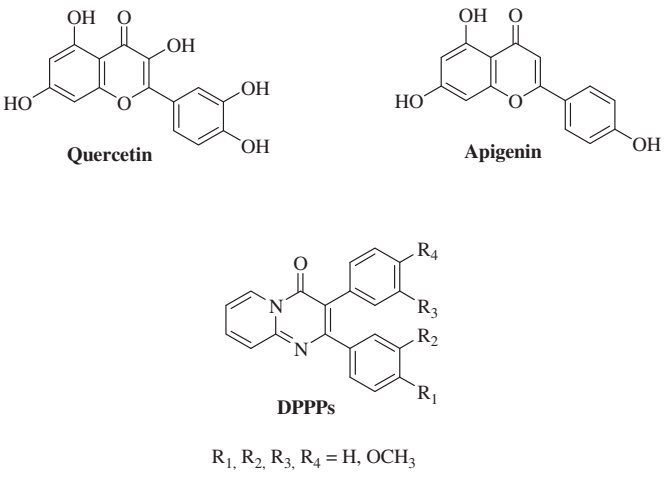


Chart 1. Quercetin, apigenin and 2,3-diphenyl-4*H*-pyrido[1,2-*a*]pyrimidin-4-ones, DPPPs.

Smooth muscle cell hyperplasia during atherosclerosis and restenosis is associated with the induction of MMP expression that remodel the surrounding extracellular matrix [32,33]. Over-expression of MMP-9 in smooth muscle cells in injured vascular wall induces considerable changes such as an increase in smooth muscle cell migration, expansive remodeling, and a decrease in extracellular matrix [32]. Therefore, the ability of our compound to also inhibit the MMP-9 up-regulation and activity can result in a lesser SMC migration and proliferation.

This study also showed that **3** reduced the cytokine-induced expression of COX-2, followed by reduction of PGE_2 production in HAoSMCs. On the contrary, in endothelial cells, **3** affected neither COX-2 protein and gene expression nor the production of prostanoids, particularly the prostacyclin PGI_2 that acts synergistically with nitric oxide to maintain normal vascular functions, i.e., atheroprotection, inhibition of platelet activation, and vascular smooth muscle contraction [34].

Since the expression of COX-2 is associated with mitogen-activated protein kinase and mediates smooth muscle cell proliferation [35,36], we suppose that the inhibitory effect of **3** on HAoSMCs cell proliferation may be due to the inhibition of COX-2 gene expression. On the other hand, the null effects of **3** on both COX-2 expression and proliferation in HUVECs, confirm the COX-2-dependent anti-proliferative response of **3** in HAoSMCs. Nonetheless, we cannot exclude other cell-signaling pathways by which **3** exerts its inhibitory effects on cell growth.

5. Concluding remarks

In conclusion, we have shown that the novel heterocyclic compound, 2-(3,4-dimethoxyphenyl)-3-phenyl-4*H*-pyrido[1,2-*a*] pyrimidin-4-one, **3**, regulates events involved in the remodeling of the vessel wall. Accordingly, it might provide a new way to modulate the vascular remodeling under pathologic conditions. Thanks to the multiple and tissue-specific function of **3**, i.e., selective down-regulation of HAoSMC proliferation, inhibition of the endothelial cell dysfunction and maintenance of the endothelial cell athero-protective action, we can assume that it could become a novel therapeutic candidate for restenosis.

This compound might replace or assist the action of current drugs eluted by coronary stents, in order to promote physiological repair of the injured wall, restoring its compatibility with the circulating blood, and thus limiting the incidence of thrombosis and restenosis after revascularization.

A better understanding of mechanisms underlying the anti-proliferative and athero-protective effects of **3** in vascular wall cells may provide strategies for improving the outcome of restenosis treatment. Animal model studies are needed to more definitively test the biological mechanisms and the actual therapeutic effectiveness of our compound.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgment

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