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Original article

A 2,3-diphenylpyrido[1,2-*a*] pyrimidin-4-one derivative inhibits specific angiogenic factors induced by TNF- α Serena Del Turco^{a,*}, Luca Quattrini^b, Rocchina Colucci^c, Melania Gaggini^a, Concettina La Motta^b, Giuseppina Basta^{a,*}^a CNR Institute of Clinical Physiology, Via G. Moruzzi, 1, 56124 Pisa, Italy^b Department of Pharmacy, University of Pisa, Via Bonanno, 6, 56126 Pisa, Italy^c Department of Pharmaceutical and Pharmacological Sciences, University of Padova, Largo Meneghetti 2, 35131 Padova, Italy

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ABSTRACT

Low-grade chronic inflammation is a key process of angiogenesis in tumour progression. We investigated whether a synthetic analogue of apigenin, the 2-(3,4-dimethoxyphenyl)-3-phenyl-4H-pyrido[1,2-*a*]pyrimidin-4-one (called DB103), interfered with the mechanisms involved in the angiogenic process induced by the inflammatory cytokine tumour necrosis factor (TNF α). In endothelial cells, DB103 but not apigenin reduced the TNF α -induced oxidative stress. DB103 inhibited the activation of ERK1/2 but not JNK, p38 and Akt kinases, while apigenin was not so selective because it inhibited essentially all examined kinases. Similarly, apigenin inhibited the TNF α -induced transcription factors CREB, STAT3, STAT5 and NF- κ B, while DB103 acted only on NF- κ B. DB103 inhibited the induced-release of angiogenic factors such as monocyte chemoattractant protein-1, interleukin-6 (IL-6) and angiopoietin-2 but not IL-8, while apigenin reduced the IL-6 and IL-8 release. DB103 revealed a better ability than apigenin to modulate proangiogenic responses induced by an inflammatory microenvironment.

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1. Introduction

It is known that chronic inflammation potentiates or promotes cancer development, growth, and progression (Yao et al., 2016). Excessive production of inflammatory cytokines stimulates proliferation of endothelial cells (ECs) culminating in sustained tumour angiogenesis (Huang and Blobel, 2016, Yao et al., 2016). The finding of high cytokine serum levels in cancer patients supported the opportunity to promote anti-inflammatory therapies in an attempt to counteract cancer progression. The tumour microenvironment is full of pro-inflammatory and proliferative factors including tumour necrosis factor (TNF)- α , interleukin (IL)-6, vascular endothelial growth factors (VEGF), metalloproteases, adhesion molecules and

chemokines, whose expression is mainly regulated by the transcription nuclear factor- κ B (NF- κ B), which plays a key role both in the inflammatory process and in the cancer development (Pikarsky et al., 2004). TNF- α is angiogenic *in vivo*, while *in vitro* it does not directly induce EC migration and proliferation but promotes the angiogenic process only in the presence of growth factors and protease (Liu et al., 2016, Salvatore et al., 2017).

An increased intake of dietary flavonoids is associated with a decreased risk of cardiovascular diseases and cancer (Hussain et al., 2016, Lewandowska et al., 2016). Flavonoids have been found to inhibit angiogenesis through multiple mechanisms including inhibition of hypoxia-signalling cascades, down-regulation of the NF- κ B pathway, and direct interaction with intracellular signalling pathways of growth factor-receptors (Hussain et al., 2016, Lewandowska et al., 2016). There is to point out that most *in vitro* studies on flavonoid antiangiogenic properties generally exploited high concentrations of specific compounds until to 100 μ mol/L (Zern and Fernandez, 2005, Romagnolo and Selmin, 2012). Hence apparent effects seen only at the limit of toxicity should be interpreted with caution. Thanks to their interesting functional properties, flavonoids have become a starting point for chemists to develop new synthetic analogues with improved functional properties than native or natural ones. Pursuing our interest

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in the synthesis of bioisosteres of flavonoids, making structural changes that have been recently described (La Motta et al., 2007), we obtained a novel class of 2,3-diphenylpyrido[1,2-a]pyrimidin-4-ones (La Motta et al., 2013, Del Turco et al., 2015). We recently

demonstrated (Del Turco et al., 2014), that, in our experimental conditions, flavonoid bioisosteres developed in our laboratory and inspired by the apigenin scaffold were well-tolerated even at 50 $\mu\text{mol/L}$, and for longer time, while the endothelial tolerability limit of apigenin for 72 h of incubation was 10 $\mu\text{mol/L}$ (Del Turco et al., 2014). In particular, we discovered that low concentrations of a synthetic derivative (2-(3,4-dimethoxyphenyl)-3-phenyl-4H-pyrido[1,2-a] pyrimidin-4-one), belonging to this class of bioisosteres and named DB103 (Chart 1), inhibited the smooth muscle cell proliferation but not the EC proliferation, and in both cell types it did not alter their vitality (Del Turco et al., 2014).

From this, it follows that its biocompatibility associated with a more specific functional activity than apigenin gives to DB103 improved functional properties. In continuity with what we have previously published, here we investigated whether DB103, compared with its natural analogue, modulated the inflammatory angiogenesis induced by TNF- α , by affecting proliferative factors and signalling pathways.

2. Material and methods

2.1. Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) except where specified. Stock solutions of apigenin and DB103 were dissolved in sterile dimethyl sulfoxide (DMSO) and stored at $-80\text{ }^{\circ}\text{C}$ at the maximum solubility of 50 mmol/L. Since the final concentration of DMSO in the culture medium never exceeded 0.1%, DMSO (0.1%) alone served as a control. At this concentration, it did not show any effect on cell viability, cell proliferation, or related molecular mechanisms (data not shown).

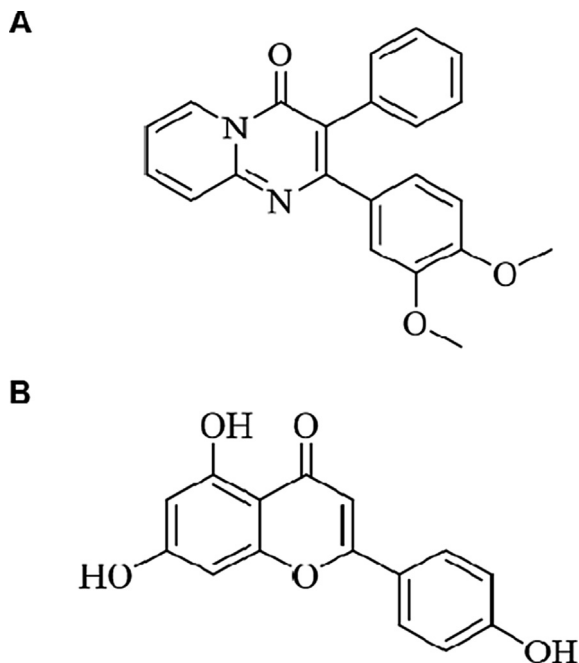


Chart 1. (A) 2-(3,4-dimethoxyphenyl)-3-phenyl-4H-pyrido[1,2-a] pyrimidin-4-one (DB103). (B) apigenin.

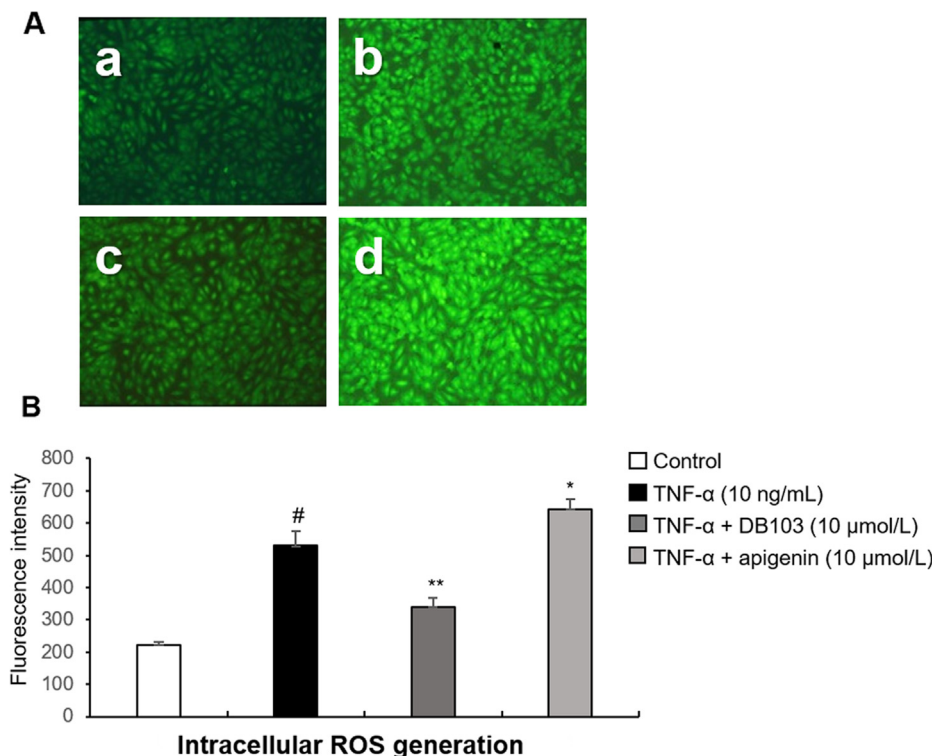


Fig. 1. DB103, but not apigenin, inhibited the TNF- α -induced intracellular ROS generation. (A) Representative photomicrographs (10x magnifications) of ROS produced in ECs incubated for 1 h with vehicle (a) or with 10 ng/mL of TNF- α (b) in the continued presence of DB103 (c) or apigenin (d) at 10 $\mu\text{mol/L}$. (B) Quantitative analysis of ROS expressed as mean \pm SD of arbitrary fluorescence units of 3 replicates. [#]P < 0.001 vs. control; ^{*}P < 0.05 and ^{**}P < 0.01 vs. TNF- α alone. Photomicrographs are representative of three experiments performed on different experimental days.

2.2. Chemical synthesis of DB103

2-(3,4-dimethoxyphenyl)-3-phenyl-4H-pyrido[1,2-a]pyrimidin-4-one, (called DB103), was synthesized from the key intermediate 2-(3,4-dimethoxyphenyl)-4H-pyrido[1,2-a]pyrimidin-4-one, prepared as reported elsewhere (La Motta et al., 2007). Briefly, the treatment of the intermediate with N-bromosuccinimide, in refluxing chloroform, produced the corresponding 3-bromo derivative that after the reaction with phenylboronic acid in the presence of bis (triphenylphosphine) palladium(II) dichloride and Na₂CO₃, in refluxing toluene, led to the target compound DB103.

2.3. Cell cultures and treatments

ECs were isolated from human umbilical vein endothelial cells, and characterized and maintained as described (Lazzerini et al., 2009). ECs 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.

If not otherwise indicated, experiments were performed on cells at pre-confluence, and cells were pre-treated for 5 min with 10 and 50 μmol/L of DB103, 10 μmol/L of apigenin or vehicle alone, before stimulation with TNF-α (10 ng/mL) for times that vary according to the experimental target. For each experimental condition, no less

than 3 replicates had been performed. Cellular toxicity by apigenin and DB103 was checked at a range of concentration from 1 μmol/L to 50 μmol/L (data not shown) through the phase contrast microscopy of cell morphology and WST-1 assay (Roche Diagnostics, Mannheim, Germany) using the manufacturers' protocol as previously described (Cervelli et al., 2014).

2.4. Detection of intracellular oxidative stress

ECs were treated with TNF-α at 10 ng/mL in the presence/absence of DB103 or apigenin for 1 h and the generation of intracellular ROS was measured with the fluorescent dye 6-carboxy-2',7' dichlorodihydrofluorescein diacetate bis(acetoxymethyl)-ester (C-DCF-DA) (Molecular Probes, Inc., Eugene, Oregon, USA) as described (Cervelli et al., 2014).

2.5. Cell signalling pathway analysis

ECs were plated in 100 mm dishes, grown at sub-confluence, and subjected to partial fetal calf serum (2%) reduction for 18 h before the addition of treatments and stimulation with TNF-α for 10 min. Cells were harvested in lysis buffer in the presence of protease inhibitor cocktail set III (Calbiochem, EMD Biosciences, San Diego, CA, USA). Each lysate (10 μg of total protein) was analysed with the Milliplex® Map Multi-Pathway 9-plex Magnetic Bead Signalling kit (Merck Millipore, Billerica, MA, USA), to detect changes

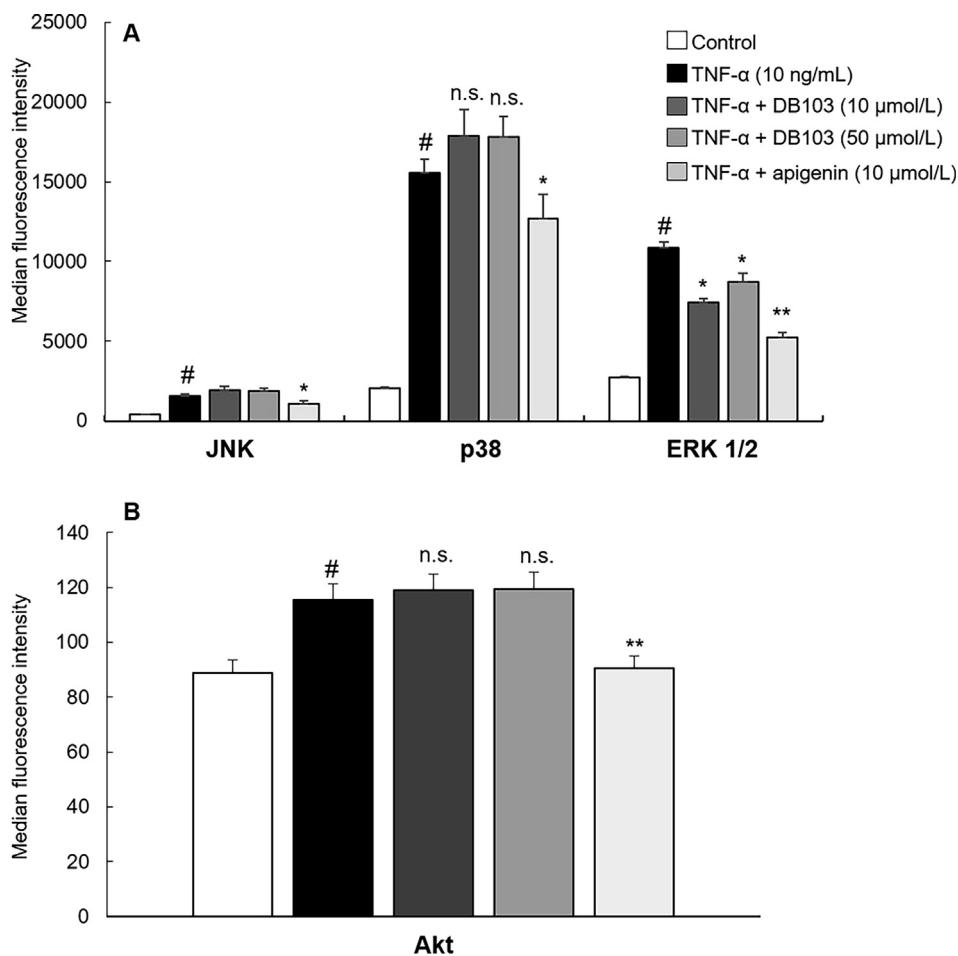


Fig. 2. Contrary to apigenin, DB103 inhibited only the TNF-α-induced ERK 1/2 kinase. ECs were incubated with vehicle or with TNF-α (10 ng/mL) in the continued presence of DB103 or apigenin. Cell lysates were assayed with multiplex technology for the phosphorylation of JNK, p38, ERK 1/2 (A) and Akt kinases (B). Results are expressed as median fluorescence intensity. Each bar represents the mean of three independent experiments, each performed in n = 4 replicates. #P < 0.001 vs. control; *P < 0.05 and **P < 0.01 vs. TNF-α alone.

in phosphorylation of the kinases [ERK 1/2 (Thr185/Tyr187), JNK (Thr183/Tyr185), p38 (Thr180/Tyr182) and Akt (Ser473)] and transcription factors [NF- κ B p65 (Ser536), STAT3(Ser727), STAT5A/B (Tyr694/699) and CREB (Ser133)], according to the manufacturer's instructions. Finally, the fluorescence intensity was read on an integrated multi-analyte detection platform (high-throughput technology MagPix system, Luminex[®] xMAP[®] technology, Merck Millipore).

2.6. ELISA analysis of the phospho-NF- κ B p65 protein

To confirm the results by the above-mentioned Luminex[®] xMAP[®] technology, the quantification of phospho-NF- κ B p65 protein was determined with the PathScan[®] Phospho-NF- κ B p65 (Ser536) Sandwich ELISA Kit (Cell Signalling Technology, Danvers, MA, USA) in the same cell lysates, according to manufacturer's

instructions. Optical density was read in a microplate reader at 450 nm.

2.7. IL-6, monocyte chemotactic protein 1 (MCP-1), VEGF-A, Angiopoietin-2(Ang-2) and IL-8 determination

After pre-treatment with the indicated compounds for 5 min and overnight stimulation with TNF- α at 10 ng/mL, culture supernatant samples were collected, frozen and thawed only once before the cytokine quantification. The amount of IL-6 and MCP-1 proteins were determined using the human IL-6 and MCP-1 Quantikine ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. The sensitivity of the IL-6 and MCP-1 was 0.7 and 1.7 pg/mL, respectively. The quantification of IL-8, VEGF-A and Ang-2 was performed with a custom-made Milliplex[®] Map Human Cytokine/Chemokine Magnetic Bead Panel (Merck Millipore). Cell culture supernatant samples were

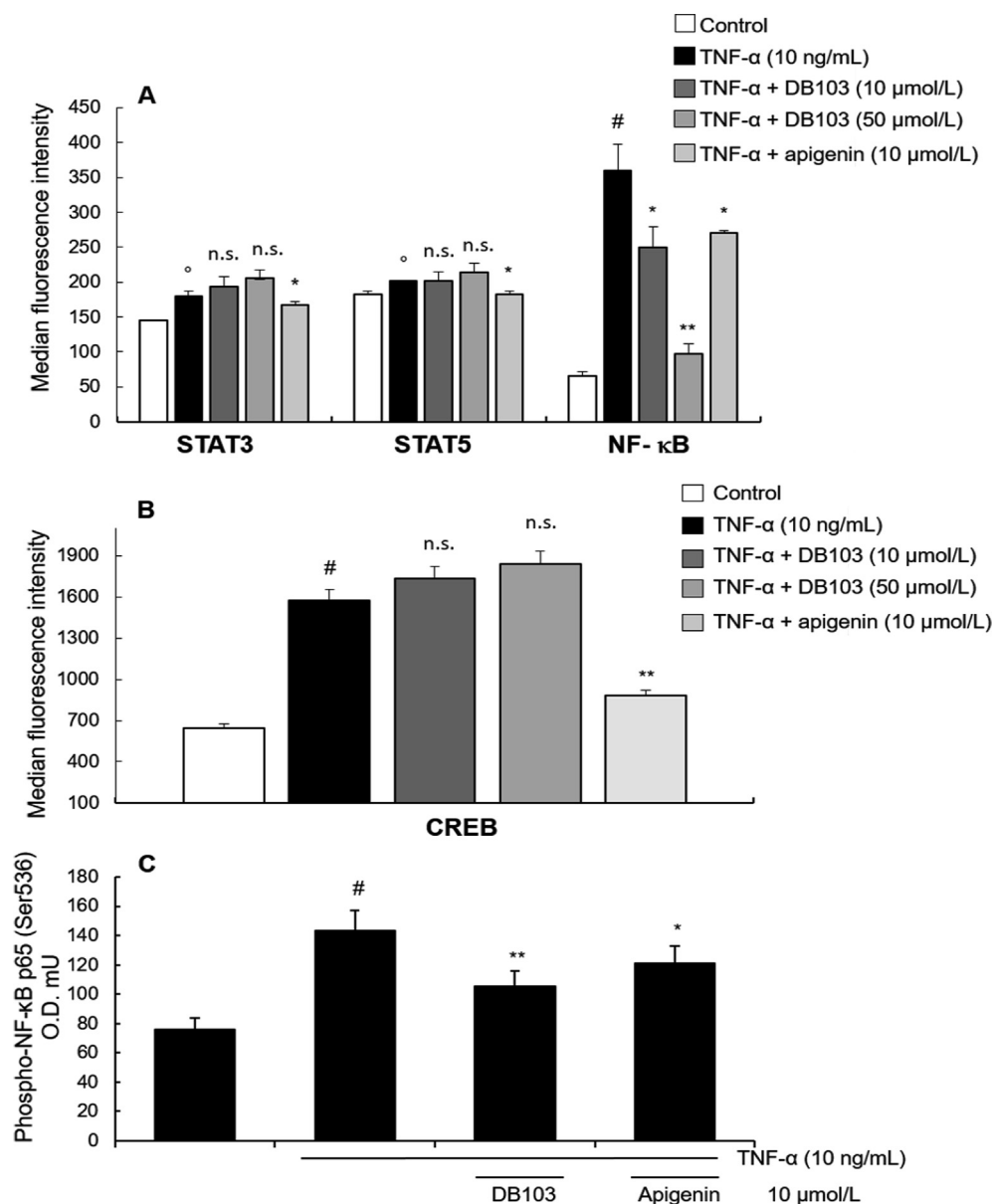


Fig. 3. DB103 inhibited only NF- κ B while apigenin inhibited indiscriminately. ECs were incubated with vehicle or with 10 ng/mL of TNF- α in the continued presence of DB103 or apigenin. Cell lysates were assayed by multiplex technology for the phosphorylation of STAT3, STAT5, NF- κ B (A) and CREB (B). Results are expressed as median fluorescence intensity. Each bar represents the mean of three independent experiments, each performed in $n = 4$ replicates. The phospho-NF- κ B p65 was also evaluated on cell lysates by an ELISA assay (C). The bars represent the mean \pm SD from four replicates. ^o $P < 0.01$ and [#] $P < 0.001$ vs. control; ^{*} $P < 0.05$ and ^{**} $P < 0.01$ vs. TNF- α alone.

processed following the manufacturer's recommended protocols and read on the Luminex® xMAP® platform (Merck Millipore). The sensitivity of VEGF-A, IL-8 and Ang-2 was 8.1, 0.2 and 3 pg/mL, respectively.

2.8. Statistical analysis

The results were expressed as mean \pm SD, percentage or median. The percentage of inhibition was calculated by the formula (value of positive control – value of sample)/value of positive con-

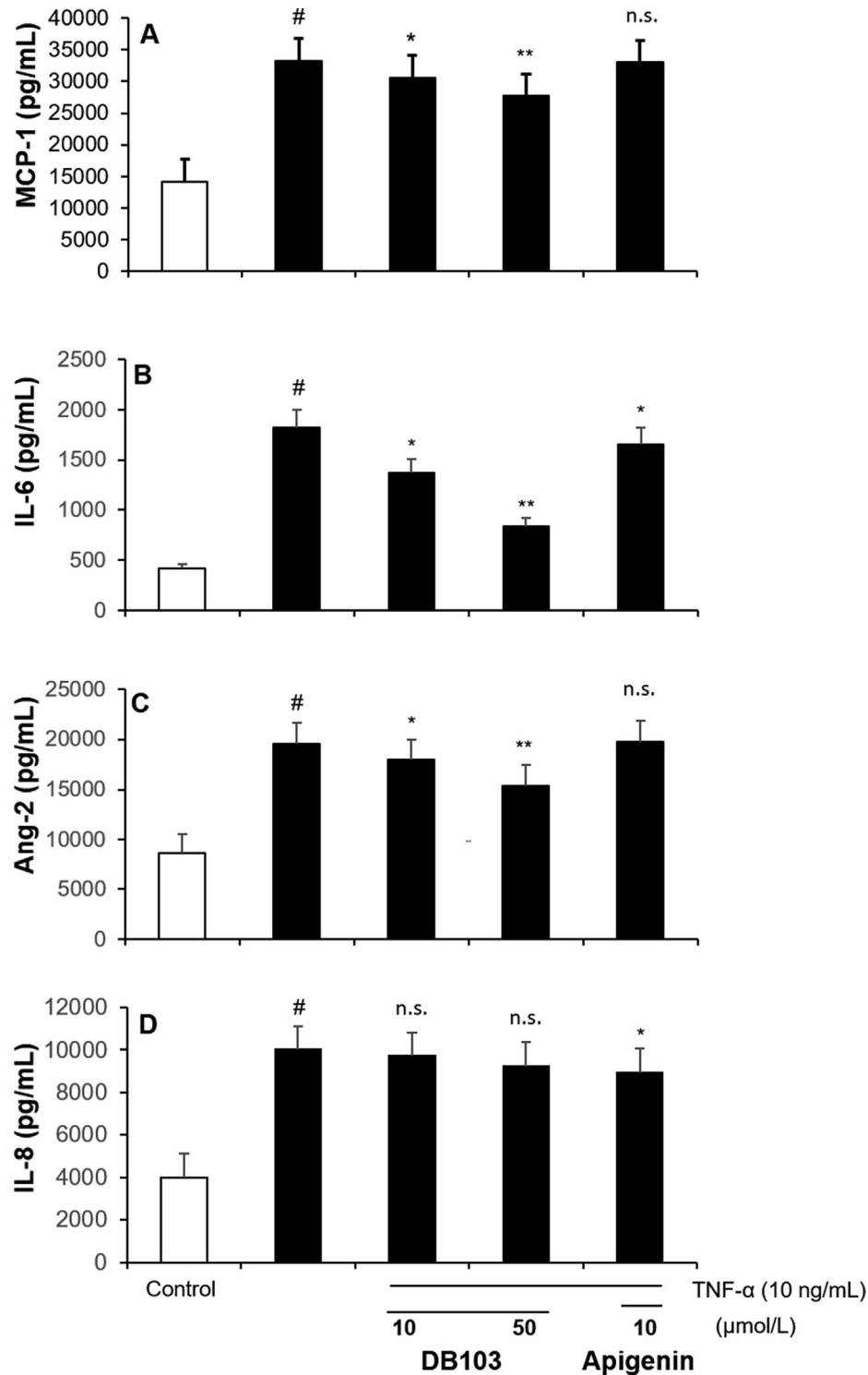


Fig. 4. DB103 inhibited IL-6, MCP-1 and Ang-2 but not IL-8 cell release. ECs were incubated for 24 h with vehicle or with 10 ng/mL of TNF- α in the continued presence of DB103 or apigenin. MCP-1 (A), IL-6 (B), Ang-2 (C) and IL-8 (D) expression were measured on cell supernatant by ELISA assay. Each bar represents the mean of three independent experiments, each performed in six replicates. ANOVA with Bonferroni's post hoc comparison. #P < 0.001 vs. control; *P < 0.05 and **P < 0.01 vs. TNF- α alone.

trol – value of negative control) × 100. 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 analysed with the use of statistical software SPSS 13.0 (SPSS Inc, Chicago, IL, USA).

3. Results and discussion

3.1. DB103 inhibited the TNF- α -induced oxidative stress

As shown in the Fig. 1, ECs stimulated for one hour with TNF- α , produced intracellular reactive oxygen species (ROS) and

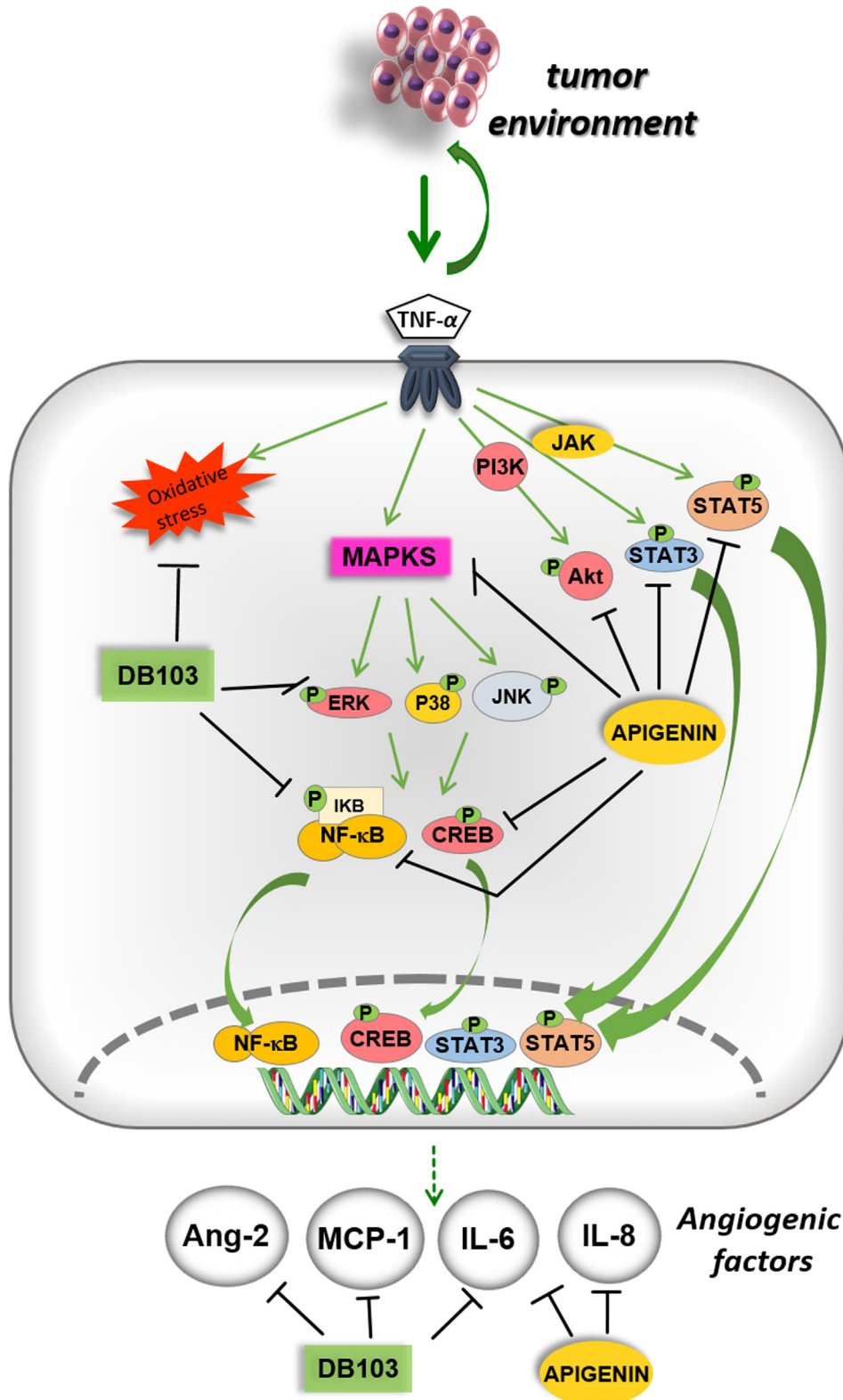


Fig. 5. Effect of DB103 and apigenin on different TNF- α signalling pathways and their molecular targets.

pre-treatment with DB103 at 10 $\mu\text{mol/L}$ strongly inhibited their production ($61.3\% \pm 10\%$ of inhibition). Pre-treatment with DB103 at 50 $\mu\text{mol/L}$ inhibited in an almost identical manner (data not shown), indicating that the maximum inhibition of ROS production was reached at 10 $\mu\text{mol/L}$. Conversely, pre-treatment with apigenin at 10 $\mu\text{mol/L}$ increased ROS burst triggered by TNF- α ($37\% \pm 7\%$ of increase) (Fig. 1).

These results suggest that DB103 has antioxidant properties that are known to be related to anti-inflammatory properties (Griffiths et al., 2016). Instead, the ROS increase by apigenin is in agreement with that recently published by Souza et al. (2017) and, as suggested by the authors themselves, could be associated to its pro-apoptotic and anti-proliferative properties (Sung et al., 2016, Souza et al., 2017).

3.2. Among the TNF- α -induced kinases, DB103 inhibited only the extracellular signal-regulated kinase (ERK) 1/2

We analysed the effects of DB103 on the main protein kinases activated by TNF- α , such as MAPKs and Akt kinase that regulate various processes including cell survival, growth and angiogenesis. After 10 min of stimulation, TNF- α activated the phosphorylation of c-Jun N-terminal kinases (JNK), p38, ERK 1/2 and Akt kinases (Fig. 2). DB103 inhibited the phosphorylation of ERK 1/2 kinase while it did not alter JNK, p38 and Akt (Fig. 2A-B). At the opposite, apigenin inhibited all examined kinases (Fig. 2A-B). These different effects on cell signalling pathways highlight that DB103 is more specific than apigenin.

3.3. Among the TNF- α -induced transcription factors, DB103 inhibited only NF- κB

We evaluated the effect of both compounds on the main transcription factors induced by TNF- α . The efficacy of apigenin was remarkable on STAT3, STAT5, NF- κB and CREB transcription factors (Fig. 3A-B) while DB103 inhibited only NF- κB phosphorylation (Fig. 3A). The phospho-NF- κB p65 induced by TNF- α was also analysed by the ELISA method, confirming the same inhibitory patterns of the two compounds on phospho-NF- κB p65 (Fig. 3C). Once again, the performance of the two compounds was dissimilar.

3.4. DB103 inhibited MCP-1, IL-6 and Ang-2 but not the IL-8 cell release

As shown in Fig. 4, upon overnight stimulation with TNF- α (10 ng/mL), ECs produced and released angiogenic cytokines, but not VEGF-A (data not shown). The absence of VEGF-A in the supernatant could be due to the fact that TNF- α -induced VEGF-A expression is not direct but it depends by other TNF- α -induced cytokines (Yoshida et al., 1997). Consequently, since its detection requires a longer time of stimulation, the overnight treatment with TNF- α resulted insufficient for a sensible detection and a following estimate of the inhibitory treatments (data not shown). DB103 inhibited MCP-1 in a concentration-dependent manner, while apigenin did not inhibit it (Fig. 4A). MCP-1, which is best known for its role in recruiting monocytes/macrophages to the arterial wall, up-regulates the hypoxia-inducible factor 1 and consequently induces VEGF-A (Hong et al., 2005) and therefore may indirectly contribute to cell proliferation and angiogenesis (Hong et al., 2005, Gacche and Meshram, 2013, Bianconi et al., 2018). Also, IL-6, which resulted to be inhibited by both DB103 and apigenin (Fig. 4B), plays a crucial role in the angiogenic process because it up-regulates the VEGF promoter activity in tumour cells (Loeffler et al., 2005, Huang et al., 2016). DB103, contrary to apigenin, reduced in a

concentration-dependent manner the TNF- α -induced release of Ang-2 (Fig. 4C), a cytokine promoting cell death that, nevertheless, combined with VEGF, can promote neo-vascularization (Hashizume et al., 2010). An opposite result was obtained about the interference of the two compounds on the TNF- α -induced release of IL-8 (Fig. 4D), a chemokine regulated by NF- κB transcription factor which activates signal transducers and activators that in turn promote angiogenesis (Vaugh and Wilson, 2008, Zhang et al., 2012). Although DB103 inhibited strongly the p65 phosphorylation, it unreduced IL-8 release (Fig. 4D), suggesting that other post-transcriptional mechanisms can be affected by DB103 with consequent absence of a net effect on production and release of IL-8 (Hoffmann et al., 2002, Wang et al., 2012). On the other hand, we cannot exclude that DB103 can exert a stimulating action and favouring the IL-8 production through other cell-signalling pathways, which we have not investigated here.

4. Conclusion

DB103 modulated the expression of several angiogenic factors induced by TNF- α as illustrated in the Fig. 5. These proliferating factors, including TNF- α , accumulate in the cancer-surrounding microenvironment and generate a low-grade, chronic inflammation, which is a crucial process involved in tumour progression (Candido and Hagemann, 2013).

DB103 revealed a superior ability than apigenin to modulate the angiogenic process induced by TNF- α .

In conclusion, although DB103 does not block the growth factors-induced physiological angiogenesis, as we previously published (Del Turco et al., 2014), it may hinder a tumour advancing by reducing factors and processes triggered by chronic and persistent inflammation of the tumour environment, characterized by the presence of cytokines and chemokines, all involved in the angiogenic development. Therefore, our compound could assist the action of current anti-proliferative drugs modulating the low-grade chronic inflammatory condition. Further studies in animal models will prove the robustness of this dietary supplement as a potential candidate for developing functional foods, utilisable to contrast the pathological angiogenic responses associated with the secretion of inflammatory cytokines in cancer.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

G. B., S.D.T designed and supervised the project. L.Q. and C.L.M. conducted the chemical synthesis of the compound. S.D.T., M.G. and R.C. performed cellular and biochemical experiments. G. B., S. D.T. and C.L.M. wrote the paper. All authors analysed and discussed the achieved results and reviewed the manuscript.

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