# Dimethylfumarate Inhibits Angiogenesis *In Vitro* and *In Vivo*: A Possible Role for Its Antipsoriatic Effect?

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The fumaric acid esters (FAEs) have been used for the oral treatment of psoriasis for some 50 years. Given that a persistent and maintained angiogenesis is associated with several cutaneous diseases, including psoriasis, we sought in our study to gain further insight into their mechanism of action by investigating whether FAEs are able to interfere with angiogenesis mechanisms. Our results demonstrate that dimethylfumarate (DMF) inhibits certain functions of endothelial cells, namely, differentiation, proliferation, and migration. This activity was not exhibited by similar concentrations of monomethylfumarate or fumaric acid. Our data indicate that DMF inhibits the growth of transformed and nontransformed cells in a dose-dependent manner. The growth-inhibitory effect exerted by this compound on proliferating endothelial cells could be due, at least in part, to an induction of apoptosis. Inhibition by DMF of the mentioned essential steps of *in vitro* angiogenesis is consistent with the observed inhibition of *in vivo* angiogenesis, substantiated using chick chorioallantoic membrane and live fluorescent zebrafish embryo neovascularization assays. The antiangiogenic activity of DMF may contribute to the antipsoriatic, antitumoral, and antimetastatic activities of this compound and suggests its potential in the treatment of angiogenesis-related malignancies.

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## INTRODUCTION

*Fumaria officinalis*, a plant rich in fumaric acid (FA), has been in use as treatment for skin complaints since the seventeenth century. The fumaric acid ester (FAE) dimethylfumarate (DMF) and its metabolite monomethylfumarate (MMF) have been used for the oral treatment of psoriasis for some 50 years. Since its official registration in 1994, the commercially available FAE preparation Fumaderm (Fumedica, Herne, Germany), an empirically composed mixture of DMF with calcium, magnesium, and zinc salts of ethylhydrogen fumarate, has become the leading oral systemic therapy for moderate to severe psoriasis in Germany (Mrowietz and Asadullah, 2005). Clinical studies, although limited in number, indicate that the efficacy of FAEs in psoriasis is high, with favorable long-term safety and clinical-efficacy profiles and relatively low toxicity (Brewer and Rogers, 2007). More recently, interest in the pharmacologic potential of FAEs has been extended to the treatment of neoplastic skin diseases. DMF has been reported to reduce melanoma growth and metastasis in murine models (Loewe *et al.*, 2006; Yamazoe *et al.*, 2009), as well as to enhance the *in vivo* antitumoral activity of the alkylating agent dacarbazine (Valero *et al.*, 2010). Q2

The mechanism of action of FAEs is not yet fully understood. In most biological assays, DMF exerts pharmacodynamic effects that are more potent than those of MMF. *In vitro* studies have revealed that DMF inhibits the expression of adhesion molecules by endothelial cells (Loewe *et al.*, 2002) and human leukocytes, affecting their rolling behavior *in vivo* and therefore the initial step of leukocyte extravasation (Rubant *et al.*, 2008). It has been shown that DMF selectively prevents the nuclear translocation of cytoplasmic NF-κB (Loewe *et al.*, 2001, 2002) and NF-κB transactivation by the p38 mitogen-activated protein kinase and the downstream kinases mitogen- and stress-activated protein kinases 1 and 2 (Gesser *et al.*, 2007). Q3 The subsequent inhibition of NF-κB-induced gene transcription may explain several of the *in vitro* effects reported for DMF on several cell types, including the inhibition of the differentiation of dendritic cells (Zhu and Mrowietz, 2001), induction of apoptosis (Treumer *et al.*, 2003; Loewe *et al.*, 2006), and inhibition of the production of proinflammatory cytokines (Ockenfels *et al.*, 1998). Recent results suggest that DMF downregulates cytokine secretion by inhibiting not only NF-κB but also a wider range of NF-κB-linked signaling proteins (Seidel *et al.*, 2009). A more detailed understanding of the mode of action of FAEs might lead to the identification of promising new drug targets. Q4

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*Abbreviations: BAEC, bovine aortic endothelial cell; CAM, chorioallantoic membrane; DMF, dimethylfumarate; EGFP, enhanced green fluorescent protein; FA, fumaric acid; FAE, fumaric acid esters(s); HUVEC, human umbilical vein endothelial cell; $IC_{50}$, half-maximal inhibitory concentration; MMF, monomethylfumarate; VEGFR2, vascular endothelial growth factor receptor 2*

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Angiogenesis is the generation of new capillaries via the sprouting pre-existing microvessels. In health, vessel proliferation is under stringent control and occurs only during embryonic development, endometrial regulation, reproductive cycle, and wound repair. By contrast, persistent and deregulated angiogenesis is related to diseases such as proliferative retinopathies, psoriasis, and rheumatoid arthritis, and it seems to be essential for tumor growth and metastasis (Carmeliet, 2005). For this reason, angiogenesis inhibition has attracted broad attention in the field of pharmacological research. Although angiogenesis research has been driven mainly by the role played by this neovascularization mechanism in tumor growth, invasion, and metastasis, the growing knowledge regarding the involvement of angiogenic factors in seemingly unrelated diseases suggests that angiogenesis can be considered an organizing principle in drug discovery, permitting connections between unrelated phenomena and enabling the development of therapeutics for one disease to facilitate the development of therapeutics for others (Folkman, 2007).

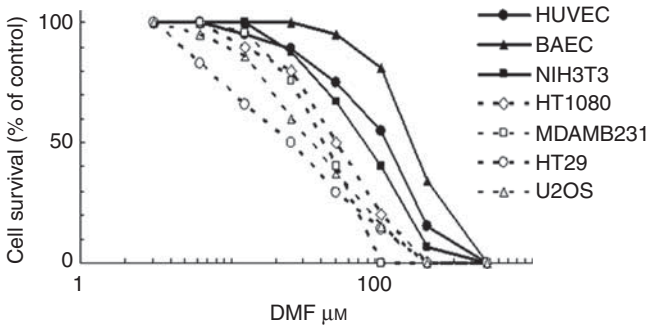
Persistent and maintained angiogenesis is associated with several cutaneous diseases, including psoriasis (Fried and Arbiser, 2008). Microvascular changes such as increased permeability, dilatation, and tortuosity of vessels—are already evident in early stages of the evolution of a psoriatic plaque (Heidenreich *et al.*, 2009; Henno *et al.*, 2010). An increasing number of angiogenesis modulators, including vascular endothelial growth factor, seem to have significant roles in the pathophysiology and may even account for the maintenance of the chronic inflammatory state (Lowes *et al.* 2007). Several antiangiogenesis strategies are currently being explored for the treatment of psoriasis, either by systemic use or by topical application (Schonthaler *et al.*, 2009; Abe *et al.*, 2010).

The possibility that the mechanism of action of some systemic therapies currently used for the treatment of psoriasis could be related to an interference in the angiogenic process cannot be discarded. In this study, we investigated the antiangiogenic activity of the antipsoriatic FAEs. To our knowledge, the results presented here are the first experimental evidence showing that DMF is a potent inhibitor of angiogenesis *in vitro* and *in vivo*. The observed antiangiogenic activity of DMF could help to explain the antipsoriatic activity of FAEs and suggest the benefits of further characterization of their pharmacological potential for the treatment of other angiogenesis-related pathologies.

## RESULTS

### DMF inhibits the growth of endothelial and tumor cells

Angiogenesis involves local proliferation of endothelial cells. Figure 1a shows that DMF inhibits the proliferation of actively growing endothelial cells in a dose–response manner. MMF and FA did not affect endothelial cell growth when tested at 200 μM (data not shown). Data obtained with non-endothelial cell lines show that DMF is not a specific inhibitor of endothelial cell growth, as the half-maximal inhibitory concentration ($IC_{50}$) values of this antiproliferative effect on endothelial cells (bovine aortic endothelial cells (BAECs) and human umbilical vein endothelial cells (HUVECs)) were in the same range of those obtained with human fibroblasts (NIH3T3). Moreover, lower $IC_{50}$ values were obtained with tumor cells (human fibrosarcoma HT-1080, human breast carcinoma MDAMB231, human colon adenocarcinoma HT29, and human osteosarcoma U2OS), suggesting a preferential activity of the compound on transformed cell lines (Figure 1b).




| | HUVEC | BAEC | NIH3T3 | HT1080 | MDAMB231 | HT29 | U2OS |
|---|---|---|---|---|---|---|---|
| $IC_{50}$ μM | 95±4 | 149±26 | 85±17 | 45±9 | 46±13 | 24±4 | 37±4 |

**Figure 1. Dimethylfumarate (DMF) inhibits the growth of endothelial and tumor cells.** (**a**) Representative dose-response curves showing the effect of DMF on the *in vitro* growth of endothelial cells (human umbilical vein endothelial cells (HUVECs) and bovine aortic endothelial cells (BAECs)), human fibroblasts (NIH3T3), and tumor cells (HT1080, MDAMB231, HT29, and U2OS). Cell survival is represented as a percentage of control cell growth in cultures containing no drug. Each point represents the mean of quadruplicates; SD values were always <10% of the mean values and are omitted for clarity. (**b**) Half-maximal inhibitory concentration ($IC_{50}$) values calculated from dose-response curves as the concentration of DMF yielding 50% of control cell survival. They are expressed as means ± SD of three independent experiments with quadruplicate samples each.

### DMF inhibits capillary tube formation by endothelial cells

The final event during angiogenesis is the organization of endothelial cells in a three-dimensional network of tubes. As shown in Figure 2a, *in vitro,* endothelial cells plated on Matrigel align themselves, forming cords that are already evident a few hours after plating. DMF was able to significantly inhibit the BAEC and HUVEC alignment and cord formation at 25 and 10 μM, respectively. Complete inhibition of endothelial morphogenesis on Matrigel was obtained at 100 μM DMF for BAECs and 50 μM DMF for HUVECs (Figure 2a and b). The DMF concentrations required to inhibit the differentiation of BAECs and HUVECs did not affect their viability after 7 hours (results not shown). MMF and FA did not significantly block tube formation by BAECs when added at 200 μM. Only partial inhibition of HUVEC tubulogenesis was observed at 200 μM MMF or FA, indicating that DMF is the most active compound in this *in vitro* assay of angiogenesis (Figure 2b).

### DMF inhibits the migratory capability of endothelial cells

Angiogenesis involves the acquisition by endothelial cells of the capability to migrate through extracellular matrix. Our data indicate that DMF produced a dose-dependent

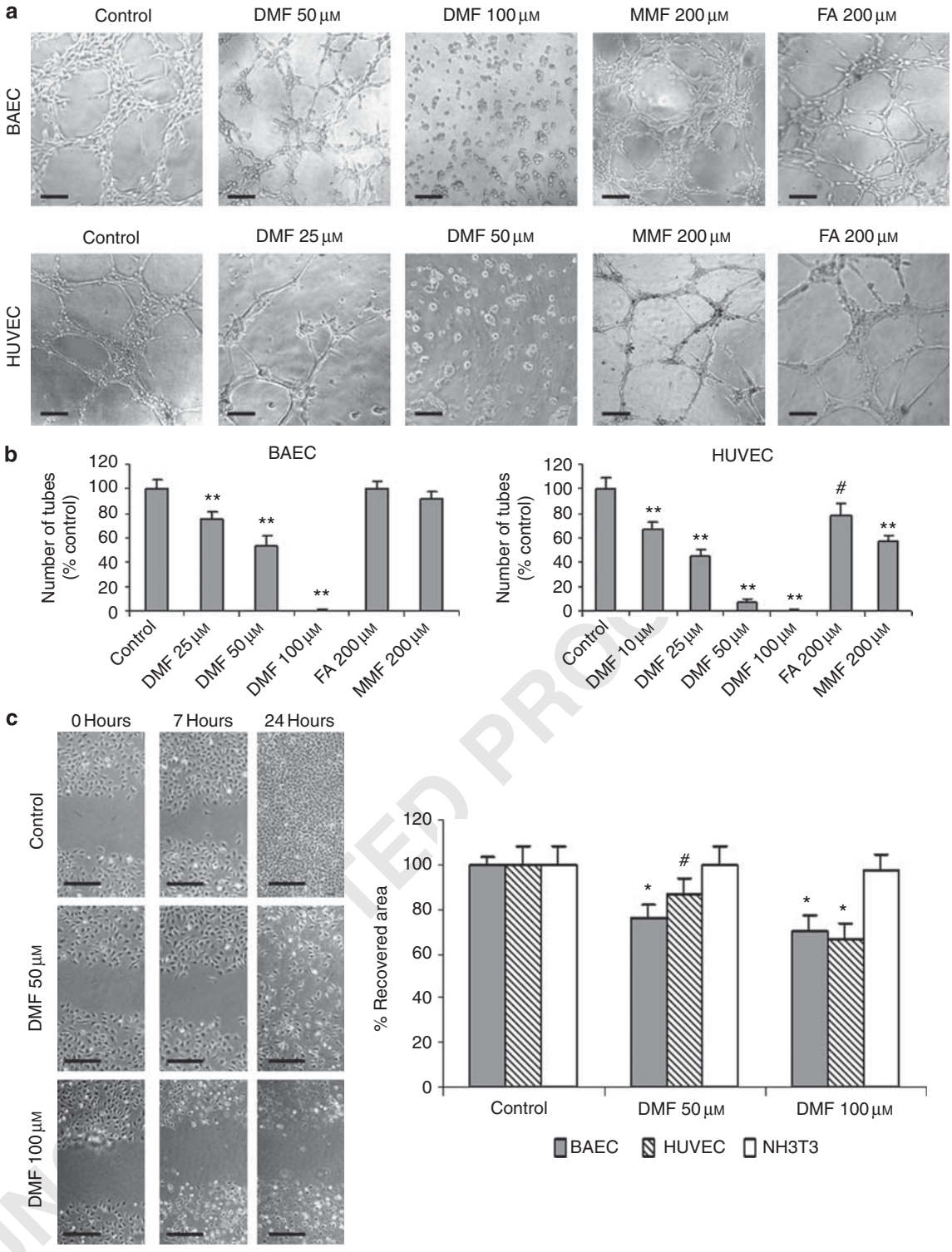



**Figure 2. Effect of fumaric acid esters (FAEs) on endothelial tube formation and cell migration *in vitro*.** (**a**) Representative images of endothelial cells treated with FAEs. Bovine aortic endothelial cells (BAECs) and human umbilical vein endothelial cells (HUVECs) seeded on Matrigel formed tubes (left panels). Dimethylfumarate (DMF) inhibited endothelial cell tubulogenesis *in vitro* at nontoxic doses. Monomethylfumarate (MMF) or fumaric acid (FA) only partially inhibited HUVEC morphogenesis on Matrigel, with no effect on BAECs, at the assayed concentration. Cells were photographed 7 hours after seeding under an inverted microscope (bar = 100 μm), and the number of tubule-like structures was counted and averaged. (**b**) Percentages of tubular-like structures are expressed as mean ± SD of three to five independent experiments, $^{**}P<0.001$, $^{\#}P<0.05$ versus control. (**c**) Confluent cell monolayers were wounded and fresh culture medium was added in either the absence or presence of the indicated concentrations of the tested compound. Photographs were taken at the beginning of the assay and after indicated times of incubation. Left panels show representative pictures of the effect of DMF on BAEC migration (bar = 200 μm). The regrowth of BAECs, HUVECs, or NIH3T cells into the cell-free area was measured after 7 hours and percentages of recovered area are expressed as mean ± SD of three independent experiments (right panel), $^{*}P<0.01$, $^{\#}P<0.05$ versus control.

inhibition of the migratory capability of endothelial cells (BAECs and HUVECs; Figure 2c and Supplementary Figure S1a online). Similar concentrations of FA or MMF did not exert this inhibitory effect on the migration of BAECs (Supplementary Figure S1b online). The effect of DMF on the migratory activity of human fibroblasts (NIH3T3) was less remarkable (Figure 2c and Supplementary Figure S1a online).

**DMF does not inhibit the kinase activity of VEGFR2**

Vascular endothelial growth factor receptor 2 (VEGFR2, KDR, Flk-1) is a major receptor for VEGF-induced signaling in endothelial cells. Upon ligand binding, VEGFR2 undergoes autophosphorylation and becomes activated. Because the kinase activity of VEGFR2 is the molecular target of a number of clinically successful angiogenesis inhibitors, we explored the effect of FAEs on this activity by means of an *in vitro* assay that directly measures the enzymatic activity of the human recombinant VEGF receptor kinase 2 on a biotinylated substrate. Our results showed that incubation with DMF, or MMF or FA, at 200 μM exerted no effect on the kinase activity of human VEGFR2 (data not shown).

**DMF induces apoptosis in endothelial cells *in vitro***

As a first approach to determine whether DMF could induce apoptosis in endothelial cells, nuclear morphology was investigated in BAECs after 14 hours of treatment with various concentrations of this compound. Figure 3a shows that 100 and 200 μM DMF induced chromatin condensation in proliferating BAECs, suggesting that DMF could induce apoptosis on endothelial cells.

To confirm this assessment, cell cycle analysis was performed in DMF-treated BAECs after propidium iodide staining. Flow cytometric analysis showed that DMF significantly increased apoptotic sub-G1 cells in a concentration-dependent manner. Thus, after treatment with DMF 100 μM for 14 hours, apoptotic cells were increased 10-fold compared with control (Figure 3b). Using the TUNEL assay as another method to detect apoptosis, DMF was shown to induce DNA fragmentation in proliferating BAECs (Figure 3c). Similar results were obtained in proliferating HUVECs (Supplementary Figure S2a online). By contrast, no significant induction of apoptosis was observed in nonproliferating quiescent BAECs (Supplementary Figure S2b online).

To determine whether caspases were activated as a result of DMF treatment, we used a caspase-3 substrate DEVD-AMC that is cleaved to a fluorescent product by caspase-3 and other caspases with similar substrate cleavage sequences. As shown in Figure 3d, the ''effector caspase''-3 was significantly activated in proliferating BAECs after treatment with DMF. Thus, the results show that caspase activation occurred in a pattern that is consistent with the DNA fragmentation and the morphological evidence of endothelial apoptosis following treatment with DMF. The results demonstrating the *in vitro* induction of apoptosis by DMF are reinforced by those showing an endothelial apoptosis induction *in vivo* by this compound in the *in vivo* quail chorioallantoic membrane (CAM) assay (Supplementary Methods and Supplementary Figure S3 online).

**DMF inhibits *in vivo* angiogenesis in the chick CAM and zebrafish embryo assays**

The CAM assay was used to determine the ability of DMF to inhibit angiogenesis *in vivo*. In controls, blood vessels formed a dense and spatially oriented, leaf-like branching network composed of vascular structures of progressively smaller diameter as they branch (Figure 4a, left panel). Table 1 summarizes the evaluation of the *in vivo* inhibition of angiogenesis in the CAM assay by DMF. Treatment with DMF caused a dose-dependent antiangiogenic effect, which was observed as an inhibition of the ingrowth of new vessels in the area covered by the methylcellulose discs. The peripheral vessels (relative to the position of the disc) grew centrifugally, avoiding the treated area, where a decrease in the vascular density could be observed (Figure 4a, right panel).

A zebrafish model system was also used to determine the effect of DMF on *in vivo* angiogenesis. The transparency and external development of the zebrafish embryo and the ability to produce tissue-specific germline transgenic fish expressing enhanced green fluorescent protein (EGFP) make this organism an ideal system with which to visualize the formation of the embryonic vasculature (Lawson and Weinstein, 2002). Different concentrations of DMF were incubated with embryos from a transgenic (*TG*(*fli1:EGFP*)*y1*) zebrafish line that carries a 15-kb promoter of the transcription factor *friend leukemia virus integration-1* (*fli-1*), which drives the GFP expression in the endothelium. As shown in Table 1, the systemic exposure of DMF exerted a dose–response inhibitory effect on the developmental angiogenesis in zebrafish. 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 4b left panel). Our results show that DMF inhibited the zebrafish intersegmental blood vessel growth and angiogenesis, although the embryos remained viable during the 24-hour period of the study and overall morphology was similar to control embryos, indicating that development was unaffected and also indicating a low toxicity of this compound (Figure 4b right panel).

The inhibition of blood circulation through the intersegmental vessels after a 24-hour incubation with DMF was further confirmed by recording the blood flow. As can be observed in Supplementary Movie S1 online, there was a continuous flow of red blood cells through the intersegmental vessels in the control zebrafish, treated with the vehicle alone. Nevertheless, when the embryos were treated with 10 μM DMF, the circulation of blood cells through the intersegmental vessel was slower than that of the control embryos (Supplementary Movie S2 online), and a complete lack of circulation with a beating heart was observed when the embryos were treated with 25 μM DMF (Supplementary Movie S3 online). In all cases, the blood flow through the aorta and posterior cardinal vein was unaffected. These results are in agreement with those obtained with the CAM assay and indicate that DMF is a potent inhibitor of *in vivo* angiogenesis.

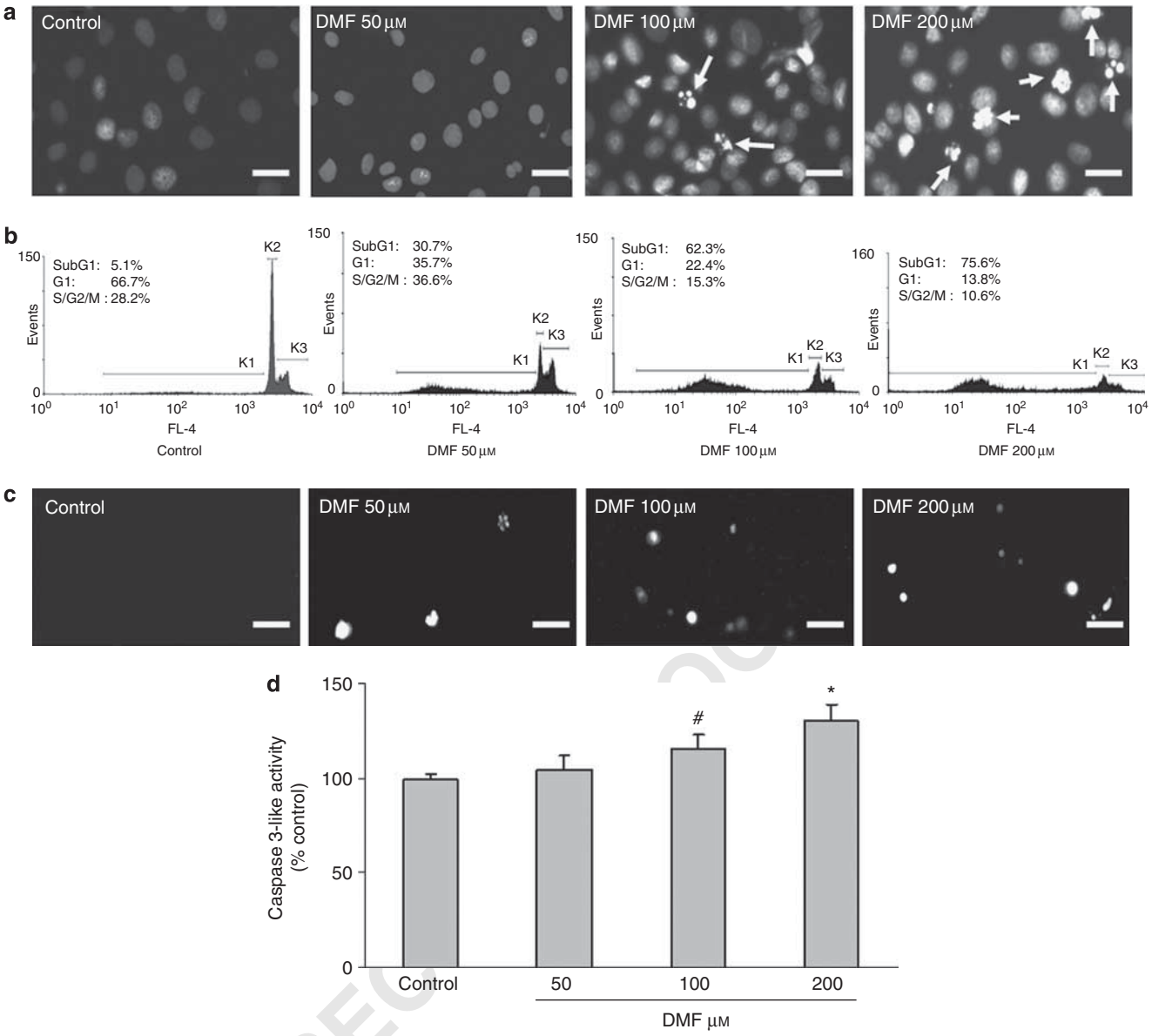



**Figure 3. Dimethylfumarate (DMF) induces endothelial cell apoptosis.** (**a**) Effect of DMF on endothelial nuclear morphology. Bovine aortic endothelial cells (BAECs) were grown on covers, treated with the indicated concentrations of DMF for 14 hours, fixed with formalin, stained with Hoechst, and mounted on slides, and nuclei were observed under a fluorescence microscope (bar = 50 μm). (**b**) Effect of DMF on endothelial cell cycle distribution. BAECs were exposed for 14 hours to DMF at the indicated concentrations and stained with propidium iodide, and percentages of subG1, G1, S, and G2/M cells were determined using a MoFlo DakoCytomation cytometer. One representative experiment of two is shown with superimposable results. (**c**) Effect of DMF on DNA fragmentation in proliferating BAE cells. BAECs, grown to 75% confluency on eight-well culture slides, were treated with the indicated concentrations of DMF for 14 hours. Then, the TUNEL assay was performed according to the manufacturer's indications (bar = 50 μm). (**d**) Effect of DMF on the caspase-3-like activity in BAECs. Cells were plated in 96-well plates and treated with the indicated concentrations of DMF for 14 hours. Then, caspase 3/7 reagent was added to wells according to the manufacturer's instructions, and the luminescence was recorded at 30 minutes with a microplate luminometer. Results are expressed as mean ± SD, $^{*}P<0.01$, $^{\#}P<0.05$ versus control.

## DISCUSSION

The FAEs have been used for the oral treatment of psoriasis for some 50 years. Because psoriasis is an angiogenesis-related pathology, and our aim was to gain further insight into the FAEs' mechanism of action, we sought to determine whether FAEs are able to interfere with angiogenesis.

Knowledge of the sequence of events required for neovascularization and the availability of cultured endothelial cells have allowed for the development and use of *in vitro* assays to resemble the different steps of the angiogenesis process and to expedite the discovery of angiogenesis inhibitors. Insofar as a factor could inhibit one or several of these key events *in vitro*, it is a candidate for the inhibition of angiogenesis *in vivo* (Quesada *et al.*, 2006).

DMF inhibits the growth of nontransformed and transformed cells in a dose-dependent manner. The $IC_{50}$ of this activity is similar for endothelial cells and normal fibroblasts, indicating that DMF is not a selective inhibitor of endothelial

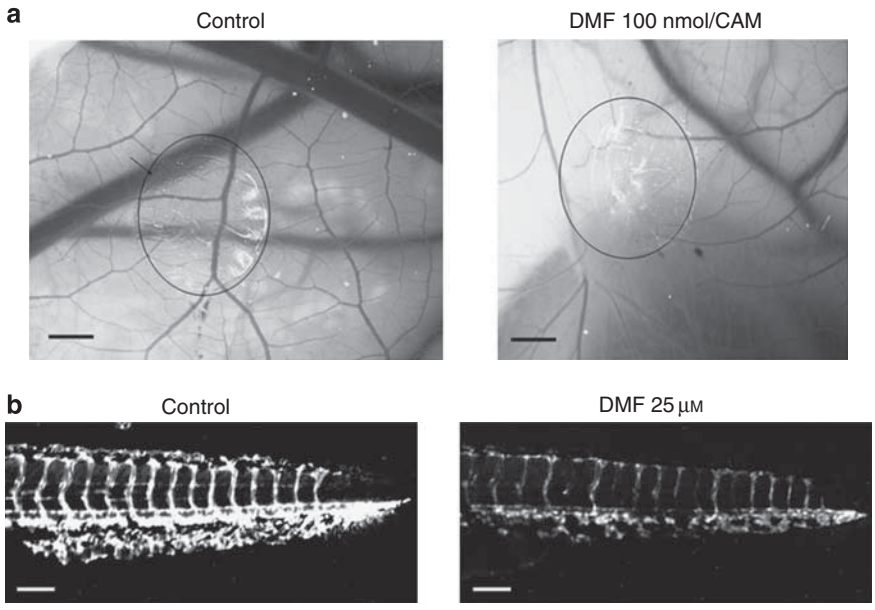



**Figure 4. Dimethylfumarate (DMF) inhibits angiogenesis *in vivo*.** (**a**) Chorioallantoic membrane (CAM) assay of DMF. (Left panel) Methylcellulose disc containing the substance vehicle alone. (Right panel) Methylcellulose disc containing 100 nmol of DMF. Circles show the locations of the methylcellulose discs (bar = 1,000 μm). (**b**) Transgenic *TGfli1:EGFPy1* zebrafish embryos, which show green fluorescent protein (GFP) expression in endothelial cells, were incubated without (left panel) or with 25 μM DMF (right panel). Blood vessel morphology was recorded by fluorescence microscopy (bar = 50 μm).

cell growth. The observation that DMF is toxic for tumor cells at concentrations at which it shows limited toxicity on normal cells suggests a potential of this compound for tumor treatment. This is in agreement with results reported for other plant-derived antitumoral compounds and suggests that metabolic and other stress conditions present in tumor cells could probably make them more sensitive to those compounds (Da Rocha *et al.*, 2001).

Our results demonstrate for the first time that DMF inhibits in a dose-dependent fashion certain functions of endothelial cells, namely, differentiation, proliferation, and migration. Inhibition of angiogenesis by DMF could not be explained by a direct inhibition of the tyrosine kinase activity of VEGFR2, as no effect was observed after *in vitro* incubation of the recombinant VEGFR2 kinase with 200 μM DMF.

The antiangiogenic activity of DMF was first detected using the *in vitro* differentiation assay for endothelial cells. Our results show that DMF is a potent inhibitor of capillary-like tube formation by BAECs or HUVECs at concentrations that are lower than their respective $IC_{50}$ values in the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. DMF also produced a dose-dependent inhibition of another key step of the angiogenic process: the migratory capability of endothelial cells. Although the little knowledge we have about the pharmacokinetics of FAEs in humans makes it difficult to extrapolate laboratory concentrations to those achievable through oral administration, the concentrations of DMF exhibiting an *in vitro* inhibition of angiogenesis are in the range of concentrations used by other authors when describing the *in vitro* activities of this compound. These activities were not exhibited by similar concentrations of MMF or FA, which is in agreement with previous results showing that in most biological assays, DMF exerts pharmacodynamic effects that are more potent than those of MMF (Mrowietz and Asadullah, 2005).

The inhibition of fibroblast cell migration by DMF was less efficient, suggesting a preferential effect of this compound on the migratory capabilities of endothelial cells. Given that

**Table 1. Inhibition of *in vivo* angiogenesis by dimethylfumarate (DMF)**

| Dose (nmol/CAM) | Positive/total | % Inhibition |
|---|---|---|
| *CAM assay* | | |
| 0 | 0/20 | 0 |
| 50 | 1/4 | 25 |
| 100 | 3/6 | 50 |
| 200 | 7/10 | 70 |
| 400 | 5/6 | 83 |
| | | |
| DMF (μM) | | |
| *Zebrafish assay* | | |
| 0 | 0/30 | 0 |
| 5 | 7/30 | 23 |
| 10 | 19/24 | 80 |
| 25 | 23/26 | 92 |

*In vivo* chorioallantoic membrane (CAM) and live fluorescent zebrafish embryo assays were carried out with different doses of DMF, as described in the Materials and Methods section. Data are given as the percentage of treated egg CAMs that showed inhibited angiogenesis or as the percentage of treated zebrafish embryos that showed inhibited angiogenesis.

previously reported results show contradictory effects of DMF on the migratory activity of other cell lines (Yamazoe *et al.*, 2009; Valero *et al.*, 2010), further experimental data regarding the comparison of the effects of DMF on several cell types and studies of the mechanisms leading to this inhibition are needed to clarify this question.

Apoptosis is associated with characteristic morphological changes, including chromatin condensation, nuclear fragmentation, cell shrinkage, plasma membrane blebbing, and the formation of apoptotic bodies. Our studies on the nuclear morphology of BAECs revealed that DMF induces nuclear changes characterized by chromatin condensation and nuclear fragmentation. This result was confirmed by monitoring of the cell cycle distribution, showing an increase in the percentage of cells with subdiploid DNA content; by TUNEL assay, showing DNA fragmentation; and by measurement of the activity of the effector caspase 3, showing activation of the caspase proteolytic cascade after treatment with DMF. Apoptosis induction was also observed by DMF treatment in proliferating HUVECs, reinforcing the hypothesis that the growth-inhibitory effect produced by DMF on proliferating endothelial cells could be due, at least in part, to an induction of apoptosis. Similarly, it has previously been reported that a number of endogenous and exogenous angiogenesis inhibitors do induce endothelial cell apoptosis, suggesting that endothelial cell apoptosis induced by a variety of mechanisms might be responsible for inhibiting angiogenesis, thereby preventing the growth of primary tumors and their metastases (Lucas *et al.*, 1998; Gururaj *et al.*, 2002; Martínez-Poveda *et al.*, 2007). Our results show that DMF induces apoptosis in proliferating endothelial cells, which could contribute to the antiangiogenic potential of this compound, and they are in agreement with the proapoptotic activity previously described for DMF in other cell types (Kirlin *et al.*, 1999; Treumer *et al.*, 2003; Mrowietz and Asadullah, 2005). The proapoptotic activity of DMF was highly reduced in nonproliferating endothelial cells, suggesting that DMF treatment could mainly affect the newly formed rather than the preexisting blood vessels and offering a possible indication of the limited toxicity of this compound.

Inhibition by DMF of the mentioned essential steps of *in vitro* angiogenesis agrees well with the observed effect on *in vivo* angiogenesis, substantiated by using two widely employed and independent experimental models: the chick CAM and the live fluorescent zebrafish embryo neovascularization assays. Our experimental data clearly show that DMF is a potent inhibitor of angiogenesis *in vivo*, with these activities being exhibited in a concentration-dependent manner.

A remarkable number of plant-derived compounds have been reported to inhibit angiogenesis *in vitro* and *in vivo* (Fan *et al.*, 2006; Bifulco *et al.*, 2007; Varinska *et al.*, 2010). Although these have widely diverse structures, some common mechanisms are noted—in particular, inhibition of the transcription factor NF-κB (De'll Eva *et al.*, 2007; Ichikawa *et al.*, 2007; Lin *et al.*, 2007). Given the relevant role of NF-κB in the control of angiogenesis (Huang *et al.*, 2000), the previously reported inhibition of NF-κB-mediated gene transcription by DMF (Loewe *et al.*, 2001 and 2002) could contribute to the observed antiangiogenic effects of this compound.

To our knowledge, the data presented here are the first direct evidence showing the antiangiogenic activity of DMF. Considering the putative role played by angiogenesis in the development of psoriasis, these data could help to explain, at least in part, the mechanism of action of the antipsoriatic activity of FAEs. Because angiogenesis not only is needed for the growth of primary tumors, but also has an essential role in metastatic spread, the observed antiangiogenic activity of DMF may also contribute to explaining the observed antitumor and antimetastatic activity of DMF (Loewe *et al.*, 2006).

Taken together, the results presented in this study show that FAEs inhibit angiogenesis *in vitro* and *in vivo*, affecting several steps of the angiogenesis process. Our findings identify DMF as the pharmacologically active compound when compared with MMF or FA regarding angiogenesis inhibition and could help to explain, at least in part, the previously described antipsoriatic, antitumoral, and antimetastatic activities of this compound. Although additional studies will be needed to elucidate the molecular mechanisms underlying the antiangiogenic activity of DMF, the data presented here suggest its potential in therapeutic applications for the treatment of angiogenesis-related malignancies.

## MATERIALS AND METHODS

### Materials

Cell culture media were purchased from Biowhittaker (Walkersville, MD). Fetal bovine serum was a product of Harlan-Seralab (Belton, UK). Matrigel was purchased from Becton Dickinson (Bedford, MA). Supplements, FAEs, and other chemicals not listed in this section were obtained from Sigma Chemicals (St Louis, MO). Plastics for cell culture were supplied by NUNC (Roskilde, Denmark).

### Cell cultures

BAECs, isolated from bovine aortic arches (Cárdenas *et al.*, 2006), and HUVECs, isolated from human umbilical cords by collagenase digestion (Kubota *et al.*, 1988), were maintained as described elsewhere (Martínez-Poveda *et al.*, 2007 and Supplementary Methods online). NIH3T3 fibroblasts and the cancer cell lines used in this study (human fibrosarcoma HT1080, human colon adenocarcinoma HT29, human osteosarcoma U2-OS, and human breast carcinoma MDA-MB-231) cells were obtained from the ATCC and maintained in culture as described by the provider (Supplementary Methods online).

### Cell growth assay

The MTT (Sigma Chemical) dye reduction assay in 96-well microplates was used as previously described (Rodríguez-Nieto *et al.*, 2002 and Supplementary Methods online). $IC_{50}$ values were calculated as the concentrations of compound yielding 50% cell survival, taking the values obtained for control as 100%.

### Tube formation on Matrigel by endothelial cells

BAE and HUVE cells were seeded on Matrigel in the presence or absence of the indicated concentrations of compounds as previously

described by us (Martínez-Poveda *et al.*, 2007 and Supplementary Methods online). After 7 hours of incubation, cultures were observed and photographed, and enclosed networks of complete tubes from randomly chosen fields were counted and averaged. Each group consisted of three or five Matrigels. For checking the viability of endothelial cells after the treatment with DMF in this assay, cells were incubated in 96-well plates in the same conditions employed for the tube formation assay. After 7 hours, cell viability in comparison with control untreated cells was determined by the addition of MTT essentially as described for the cell growth assay.

### Endothelial cell migration assay

The migratory activity of BAE, HUVE, and NIH3T3 cells was assessed using a wounding migration assay. Confluent monolayers were wounded and cells were supplied with complete medium in the absence (controls) or presence of different concentrations of FAEs. Wounded areas were photographed at different times of incubation, and the amount of migration at 7 hours was determined by image analysis in both controls and treated wells and normalized to their respective values at zero time, using the NIH Image 1.6 software (Martínez-Poveda *et al.*, 2007 and Supplementary Methods online).

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### Apoptosis assays

After treatment with the indicated concentrations of DMF for 14 hours, apoptosis assays were carried out by staining of nuclei with Hoechst, cell cycle analysis by flow cytometry, and TUNEL assay as we have described elsewhere (Martínez-Poveda *et al.*, 2007 and Supplementary Methods online).

For the determination of caspase 3/7 activity, Caspase-Glo 3/7 reagent (Promega Biotech Ibérica, Madrid, Spain) was used according to the manufacturer's instructions (Supplementary Methods online).

### *In vivo* CAM assay

The *in vivo* chicken CAM assay was carried out as described elsewhere, using fertilized chick eggs provided by Granja Santa Isabel (Cordoba, Spain) (Rodríguez-Nieto *et al.*, 2002 and Supplementary Methods online).

### Zebrafish embryo assay

Zebrafish embryos were generated via natural pairwise mating and maintained in embryo water at 28.5 °C. Transgenic *Fli-EGFP* fish (*TGfli1:EGFPy1*) had a vasculature labeled with GFP and were purchased from the Zebrafish International Resource Center (ZIRC, Eugene, OR) (Lawson and Weinstein, 2002). Embryos were manually dechorionated with forceps at 24 hour postfertilization, arrayed in a 96-well plate (one embryo per well), and incubated with 100 µl of the indicated concentrations of DMF at 28.5 °C for 24 hours. DMSO was used as both carrier of drugs and control. After incubation, fish embryos were anesthetized with tricaine (0.02%), placed on slides, and examined under an epifluorescence Nikon microscope equipped with a DS-L1 Nikon digital camera. Phenotypic changes were evaluated by two separate observers. Movies of the flow of blood through the intersegmental vessels were made 24 hours after incubation with the drugs, with a LEICA DMIL inverted microscope at low and high magnification ( $\times 4$ and $\times 20$).

Q12

### *In vitro* VEGFR2 kinase inhibition assay

VEGFR2 inhibition assay was performed using an HTScan VEGFR2 kinase kit (Cell Signaling Technology), combined with colorimetric ELISA detection, according to the supplier's instructions (see Supplementary Methods online).

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### Statistical analysis

Results are expressed as mean ± SD. Statistical significance was determined using the two-sided Student *t*-test. Values of $P<0.05$ were considered to be statistically significant.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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