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RESEARCH PAPER

Anti-angiogenic properties of a sulindac analogue

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Background and purpose: Angiopoietins (Ang) are crucial for new blood vessel formation and exert their effects by acting on the Tie2 receptor. We have recently described a sulindac analogue 2-((1E,Z)-1-benzylidene-5-bromo-2-methyl-1H-inden-3-yl)acetic acid; termed C-18 from now onwards) that inhibits Tie2 receptor activity in kinase assays *in vitro*. Here, we have assessed the ability of C-18 to inhibit angiogenesis-related properties of endothelial cells and tested its selectivity for the Tie2 receptor.
Experimental approach: For *in vitro* experiments human umbilical vein endothelial cells (HUVEC) were used. Proliferation was measured using the MTT assay; migration assays were performed in a modified Boyden chamber and tube-like structure formation was determined on matrigel. The effects of C-18 *in vivo* were evaluated in the chicken chorioallantoic membrane (CAM).
Key results: Pre-treatment of HUVEC with C-18 blocked Ang-1-stimulated migration, but also abolished vascular endothelial cell growth factor (VEGF)- and fibroblast growth factor 2-induced responses. Incubation with C-18 inhibited serum-induced proliferation in a concentration-dependent manner; C-18 was, however, without effect on Ang-1-induced survival. In addition, we observed that C-18 did not inhibit ligand-induced receptor phosphorylation of Tie2 or VEGFR2. On the other hand, C-18 blocked activation of members of the mitogen-activated protein kinase family and of the Ser/Thr kinase Akt induced by both VEGF and Ang-1. Furthermore, incubation of CAMs with C-18 led to a dose-dependent inhibition of vascular length.
Conclusions and implications: C-18 did not act as a Tie2 inhibitor, as originally thought, but rather inhibited growth factor-stimulated signalling pathways that regulate endothelial cell migration and potently reduces neovascularization *in vivo*.
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Keywords: angiogenesis; Tie2; angiopoietin; sulindac; migration; MAPK

Abbreviations: Ang, angiopoietin; C-18, 2-((1E,Z)-1-benzylidene-5-bromo-2-methyl-1H-inden-3-yl)acetic acid; CAM, chicken chorioallantoic membrane; ERK, extracellular signal regulated kinase; FGF, fibroblast growth factor; HUVEC, human umbilical vein endothelial cell; MAPK, mitogen-activated protein kinase; RTK, receptor tyrosine kinase; sGC, soluble guanylyl cyclase; SNP, sodium nitroprusside; VEGF, vascular endothelial growth factor

Introduction

Angiogenesis is a tightly regulated, multistep process that involves endothelial cell proliferation, migration and organization into capillary structures (Folkman and Shing, 1992; Conway *et al*., 2001; Carmeliet, 2003). Among the many soluble and matrix-derived angiogenic growth factors and regulators of angiogenesis that contribute to neovascularization, vascular endothelial growth factor (VEGF) and the angiopoietins (Angs) are crucial for new blood vessel formation (Yancopoulos *et al*., 2000). VEGF acts on the kinase insert domain-containing receptor (KDR) to stimulate growth, migration and permeability of endothelial cells (Ferrara *et al*., 2003). On the other hand, Ang binds and modulates the activity of the Tie2 receptor (Jones *et al*., 2001). The best-studied member of the Ang family is Ang-1. Activation of Tie2 by Ang-1 has been linked to promotion of endothelial cell survival, induction of migration and sprouting (Koblizek *et al*., 1998; Witzenbichler *et al*., 1998; Papapetropoulos *et al*., 2000). In addition, Ang-1 stimulates the recruitment of mural cells to the newly formed vessels

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providing the necessary structural support (Davis *et al*., 1996; Holash *et al*., 1999). However, Ang-2 acts as a context-dependent antagonist of Tie2, promoting vessel plasticity (Maisonpierre *et al*., 1997). Increased Ang-2 levels, lead to destabilization of vascular structures and allow other growth factors, like VEGF, to exert their angiogenic effects (Davis and Yancopoulos, 1999). Upregulation of Ang-2 production in the absence of VEGF is thought to lead to vessel regression.

Much of the progress in our understanding of the biology and functions of the Ang/Tie2 system has come from genetic studies (Sato *et al*., 1995; Suri *et al*., 1996, 1998; Maisonpierre *et al*., 1997; Thurston *et al*., 1999; Gale *et al*., 2002). Additional strategies for interfering with Ang/Tie2 signalling, which have expanded our knowledge on the role of angiopoietins in new blood vessel formation, include the use of soluble Tie2 (Lin *et al*., 1997), RNA aptamers (White *et al*., 2003) and siRNA for the angiopoietins or Tie2 (Daly *et al*., 2006; Parikh *et al*., 2006). However, more detailed studies designed to address the contribution of Tie2-regulated pathways in physiology and disease have been hampered by the lack of selective small molecule inhibitors for this receptor tyrosine kinase (RTK). We have recently reported on the synthesis of a series of sulindac analogues that inhibit the enzymatic activity of RTKs for angiogenic growth factors (Gourzoulidou *et al*., 2005). One of them, 2-((1E,Z)-1-benzylidene-5-bromo-2-methyl-1H-inden-3-yl)acetic acid (C-18; Figure 1), was found to selectively inhibit Tie2 *in vitro* with an IC_{50} in the low micromolar range. C-18 was later shown to resensitize retinal vessels *in vivo* (Hoffmann *et al*., 2005); this was proposed to result from Tie2 inhibition as administration of soluble Tie2 exerted similar effects. C-18 is, thus, beginning to emerge in the literature as a small molecule inhibitor for Tie2.

The aim of the present study was to characterize this Tie2 inhibitor pharmacologically by determining the ability of C-18 to block Ang-1-stimulated signalling and to evaluate its effects on angiogenesis-related properties of cultured endothelial cells triggered by a variety of growth factors. Moreover, we have evaluated the effects of C-18 on neovascularization *in vivo* using the chicken chorioallantoic membrane (CAM) assay.

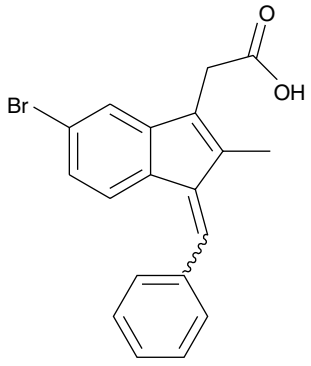

Figure 1 Chemical structure of 2-((1E,Z)-1-benzylidene-5-bromo-2-methyl-1H-inden-3-yl)acetic acid (C-18).

Materials and methods

Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from 3–4 fresh cords and grown on 100-mm dishes in M199 supplemented with 15% fetal calf serum, 50 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin, 50 μg ml^{-1} gentamycin, 2.5 μg ml^{-1} amphotericin B, 5 U ml^{-1} sodium heparin and 150–200 μg ml^{-1} endothelial cell growth supplement. Cells were used at the first or second passage.

Cell migration

Cells were serum-starved overnight. To inhibit Tie-2, cells were pretreated with C-18 for 15 min prior to trypsinization. After trypsinization, 1×10^5 cells were added to transwells (8 μM pore size) in 100 μl of starvation medium containing C-18. C-18 was also added to the well containing the transwell inserts in 600 μl volume along with the following agents: Ang-1 (250 ng ml^{-1}), Ang-2 (250 ng ml^{-1}), fibroblast growth factor (FGF)-2 (10 ng ml^{-1}), VEGF (50 ng ml^{-1}), BAY 41-2272 (0.1 μM) or vehicle (dimethylsulphoxide (DMSO)). HUVECs were allowed to migrate for 4 h at 37 °C, and after this time, nonmigrated cells at the top of the transwell filter were removed with a cotton swab. The migrated cells were fixed in Carson's solution for at least 30 min at room temperature and then stained in toluidine blue for 20 min at room temperature. Migrated cells were scored in eight random fields and the fold change was determined relative to the number of migrated cells in control wells.

Caspase-3 activity

Human umbilical vein endothelial cells were plated in 12-well cell culture plates and when confluent were switched to serum-free medium. After pretreatment with vehicle or C-18 for 30 min, cells were exposed to Ang-1 (250 ng ml^{-1}) for 24 h. At the end of this incubation period, both floating and adherent cells were collected, and caspase-3 activity was determined by measuring the proteolytic cleavage of the fluorogenic substrate Z-DEVD-AMC. To do so, cells were lysed in a buffer containing 10 mM Tris pH7.5, 100 mM NaCl, 1 mM EDTA and 0.01% Triton X-100. The fluorescence of the cleaved substrate was measured at 380-nm excitation and 469-nm emission, 30 min after the addition of 100 μM substrate to 20–50 μg of protein. Data were expressed in relative fluorescent units after normalization for protein content.

Cell proliferation

Human umbilical vein endothelial cells were seeded in 24-well plates at 6×10^3 cells per cm^2 in growth medium. They were then incubated with the indicated concentration of C-18 or vehicle (DMSO) and allowed to proliferate for 48 h. Cell proliferation was measured using the MTT colorimetric assay. In all assays, performed cell viability was >97% as measured by Trypan blue exclusion.

Capillary-like morphogenesis
Formation of endothelial tube-like structures was assessed using growth factor-reduced Matrigel matrix. HUVEC were plated at 15 000 cells per well in 96-well plates that were pre-coated with 45 μl of Matrigel in the presence or absence of C-18 (1 nM). After 24 h of incubation, tube-like structure formation was quantified using image analysis software.

Western blotting
Following treatments cellular proteins were extracted in a buffer containing 1% Triton X-100, 1% SDS, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1 mM EGTA and protease inhibitors (10 μg ml^{-1} aprotinin, 10 μg ml^{-1} pepstatin and 20 mM phenylmethylsulphonyl fluoride (PMSF)). Samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to a polyvinylidene difluoride membrane and incubated with the indicated primary and appropriate secondary antibodies. Immunoreactive proteins were detected using a chemiluminescent substrate.

Immunoprecipitations
For immunoprecipitation, 250–300 μg of cell lysates were incubated with 2 μg of polyclonal anti-Tie-2 antibody and protein G-conjugated agarose beads overnight at 4 °C. To reduce nonspecific binding, the protein G beads were preincubated with BSA for 1 h at 4 °C and washed twice with lysis buffer. The precipitated proteins were resolved in a 6% gel and transferred to a polyvinylidene difluoride membrane. Membranes were then subjected to western blotting with the appropriate antibodies.

CAM angiogenesis assay
Fertilized White Leghorn chicken eggs were placed in an incubator as soon as embryogenesis started and kept under constant humidity at 37 °C. On day 4, a square window was opened in the shell and then sealed with adhesive tape. On day 9, an O-ring (1 cm^2) was placed on the surface of the CAM, and C-18 or vehicle (10^{-4}% DMSO in phosphate-buffered saline) was added inside this restricted area. The indicated dose of C-18 was added on the CAM as a solution of a final volume of 40 μl. After 48 h, CAMs were fixed in Carson's solution (saline-buffered formalin), and angiogenesis was evaluated using image analysis software. For the CAM experiments, 30–50 eggs per group were analysed.

Data analysis
Data are expressed as means ± s.e.mean of the indicated number of observations. Statistical comparisons between groups were performed using ANOVA followed by a *post hoc* test or Student's *t*-test, as appropriate. Differences were considered significant when $P<0.05$.

Materials
Cell culture media and serum were obtained from Life Technologies GIBCO-BRL (Paisley, UK). All cell culture plastic ware was purchased from Corning-Costar Inc. (Corning, NY, USA); SuperSignal West Pico chemiluminescent substrate from Pierce Biotechnology (Rockford, IL, USA); DC Protein assay kit, Tween 20 and other immunoblotting reagents were obtained from Bio-Rad Laboratories (Hercules, CA, USA); penicillin and streptomycin from Applichem (Darmstadt, Germany); amphotericin, gentamycin and heparin were purchased from Biochrom AG (Berlin, Germany). All other reagents including BSA, EDTA and protease inhibitors were purchased from Sigma-Aldrich (St Louis, MO, USA). C-18 was synthesized as described by Gourzoulidou *et al*. (2005). VEGF, Ang-1 and Ang-2 were purchased from R&D (Minneapolis, MN, USA). FGF-2 was from Peprotech (London, UK). The EnzCheck kit for caspase-3 determination was obtained from Molecular Probes (Eugene, OR, USA). The Tie2 antibody was from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA). Extracellular signal regulated kinase (ERK) 1/2, p38, Akt, VEGR2, Tie-2 phospho-specific and total antibodies along with the secondary antibodies were obtained from Cell Signalling Technology (Beverly, MA, USA). Fertilized White Leghorn chicken eggs were obtained from Pindos (Iperos, Greece).

Results

C-18 inhibits Ang-1-induced migration, but has no effect on Ang-1-stimulated survival
Endothelial migration plays a central part in the process of angiogenesis. In line with its pro-angiogenic profile, we and others have shown that Ang-1 acts as a chemoattractant for endothelial cells (Witzenbichler *et al*., 1998; Papapetropoulos *et al*., 1999). To study whether the sulindac analogue (Figure 1) inhibits Ang-1-stimulated migration, HUVEC mobility was evaluated in a modified Boyden chamber assay. C-18 exhibited no effect on basal endothelial migration, but it abolished Ang-1-stimulated migration (Figure 2a). This inhibitory effect of C-18 was observed at 100 pM, a concentration well below its reported IC_{50} for *in vitro* kinase assays (Gourzoulidou *et al*., 2005).

To study whether C-18 in addition to blocking Ang-1-induced migration can also inhibit the pro-survival action of Ang-1, HUVEC were serum-deprived and incubated with Ang-1 in the presence or absence of the sulindac analogue (Figure 2b). In agreement to previous reports, Ang-1 reduced the rate of HUVEC apoptosis as reflected by the inhibition of caspase-3 activity; however, C-18 was unable to reverse the antiapoptotic action of Ang-1.

C-18 blocks serum-induced proliferation
In addition to increased cell motility, endothelial cell proliferation is a prerequisite for new blood vessel formation, and many agents that inhibit neovascularization do so by blocking endothelial cell growth. Although some studies have reported that Ang-1 is mitogenic, in our hands, Ang-1 lacks growth-stimulatory effects (Papapetropoulos *et al*.,

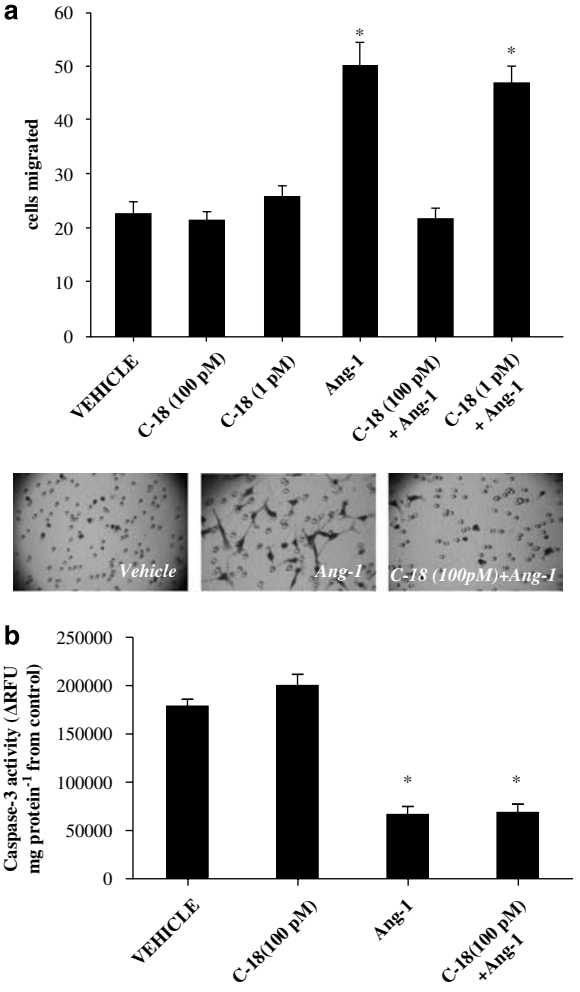

Figure 2 C-18 inhibits Ang-1-induced human umbilical vein endothelial cell (HUVEC) migration. (**a**) Cells were placed in transwells and allowed to migrate for 4 h in the presence of C-18 (1 or 100 pM), Ang-1 (250 ng ml^{-1}) or both. Migrated HUVEC were fixed, stained and counted in eight random fields. Data are expressed as means ± s.e.mean; $n=5$; *$P<0.05$ vs vehicle. Representative photomicrographs of migrated cells at × 100 magnification (bottom). (**b**) HUVECs were serum-starved, and Ang-1 (250 ng ml^{-1}) was added as a survival factor in the presence or absence of C-18 (100 pM). After 24 h, caspase-3 activity was determined as an index of apoptosis according to the manufacturers' instructions. Data are expressed as means ± s.e.mean; $n=4$; *$P<0.05$ vs vehicle. Ang, angiopoietin; C-18, 2-((1E,Z)-1-benzylidene-5-bromo-2-methyl-1H-inden-3-yl)acetic acid.

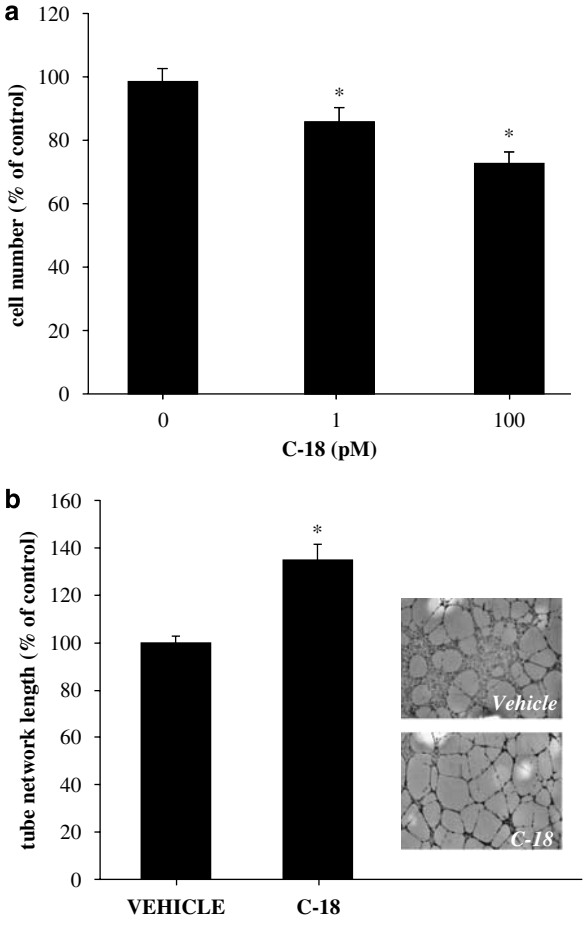

Figure 3 Effect of C-18 in proliferation and capillary morphogenesis. (**a**) Human umbilical vein endothelial cells (HUVECs) were seeded in 24-well plates, treated with C-18 (1 or 100 pM) or vehicle (dimethylsulphoxide (DMSO)) and allowed to proliferate for 48 h. Cell number was measured using the MTT colorimetric assay. Data are expressed as means ± s.e.mean; $n=16$; *$P<0.05$ vs control. (**b**) HUVECs were cultured on growth factor-reduced matrigel in the presence of C-18 (1 nM) or DMSO (control) for 24 h and photographed. Vessel-like network length was determined using image analysis software. Data are expressed as means ± s.e.mean; $n=6$; *$P<0.05$ vs vehicle. Representative photomicrographs of control and C-18-treated cultures (right). C-18, 2-((1E,Z)-1-benzylidene-5-bromo-2-methyl-1H-inden-3-yl)acetic acid.

1999). Therefore, to determine the effect of C-18 on proliferation, we studied growth of HUVEC in the presence of serum. Indeed, C-18 reduced HUVEC proliferation reaching a maxima reduction of approximately 30% at 100 pM (Figure 3a). It should be noted that at higher concentrations, C-18 was no longer effective in reducing HUVEC growth, exhibiting a bell-shaped concentration–response curve (data not shown).

Effect of C-18 on matrix-driven capillary morphogenesis

To determine whether C-18 affects the ability of endothelial cells to form tube-like network structures *in vitro*, we assessed the effects of C-18 on Matrigel-driven network formation (Figure 3b). Contrary to what would be expected for an angiogenesis inhibitor, C-18 increased total length of vessel-like structures.

C-18 inhibits VEGF- and FGF-2-induced migration

Next, we sought to determine whether this sulindac analogue affects EC motility in response to growth factors other than Ang-1. For these experiments, FGF-2 and VEGF were used; both these agents stimulated a 3.5- and 4.5-fold increase in migration, respectively, under the conditions used (Figure 4). Pretreatment with C-18 (100 pM) abolished the migratory effects of both growth factors, reducing the number of migrating cells to basal levels.

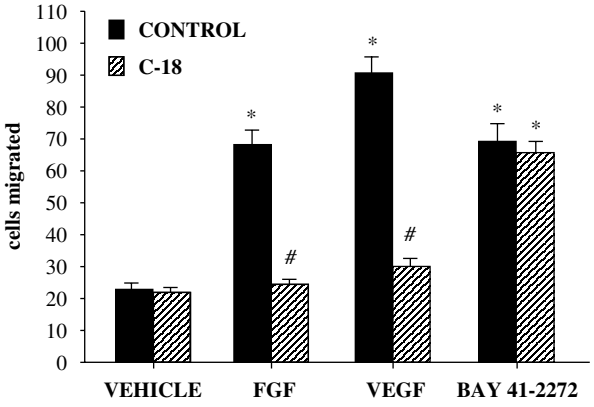

Figure 4 C-18 inhibits human umbilical vein endothelial cell (HUVEC) migration in response to a variety of growth factors. Cells were placed in transwells and allowed to migrate for 4 h in the presence of fibroblast growth factor (FGF)-2 (10 ng ml^{-1}), vascular endothelial growth factor (VEGF; 50 ng ml^{-1}) or BAY 41-2272 (0.1 μM) with or without C-18 (100 pM). Migrated HUVECs were fixed, stained and counted in eight random fields. Data are expressed as means ± s.e.mean; $n=5$; *$P<0.05$ vs vehicle; $^{\#}P<0.05$ vs control. C-18, 2-((1E,Z)-1-benzylidene-5-bromo-2-methyl-1H-inden-3-yl)acetic acid.

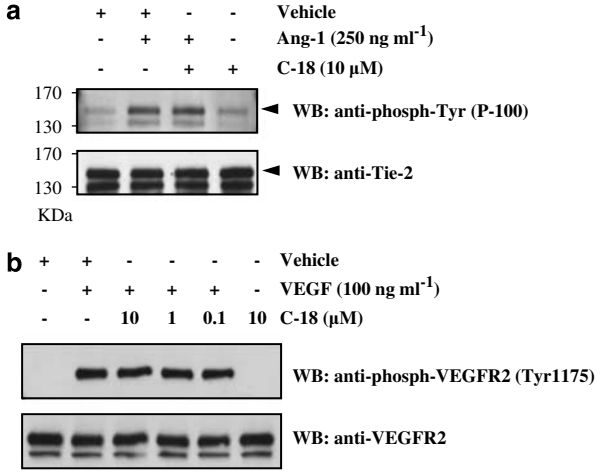

Figure 5 C-18 does not inhibit receptor tyrosine kinase (RTK) phosphorylation. (**a**) Human umbilical vein endothelial cells (HUVECs) were incubated with Ang-1 (250 μg ml^{-1}) for 5 min in the presence or absence of C-18 (10 μM; 30 min pretreatment). Cells were then lysed and the Tie2 receptor precipitated; after SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transfer, membranes were blotted with a phosphotyrosine Ab or a Tie2 antibody. Similar results were obtained when C-18 was used at 1 nM. (**b**) HUVECs were incubated with vascular endothelial growth factor (VEGF; 100 μg ml^{-1}) for 2 min with or without pretreatment of the indicated C-18 concentration. Cells lysates were analysed using total or phospho-specific (Tyr-1175) VEGFR2 antibodies. Blots shown are representative of experiments repeated twice with similar results. Ang, angiopoietin; C-18, 2-((1E,Z)-1-benzylidene-5-bromo-2-methyl-1H-inden-3-yl)acetic acid.

We and others have previously shown that the VEGF-stimulated migration is mediated by activation of soluble guanylyl cyclase (sGC) and cGMP generation (Papapetropoulos *et al*., 1997; Ziche *et al*., 1997). To examine whether C-18 acts upstream or downstream of sGC in inhibiting migration, we determined its ability to interfere with HUVEC migration triggered by BAY 41-2272, an sGC activator (Figure 4). In agreement with our recent work (Pyriochou *et al*., 2006), treatment of cells with the sCG activator BAY 41-2272 (0.1 μM) resulted in a 3-fold increase in the number of migrating cells. Interestingly, pretreatment with C-18 had no effect on BAY 41-2272-induced migration. These findings suggest that inhibition of VEGF-induced signalling by C-18 occurs upstream of sGC.

Effects of C-18 on receptor tyrosine kinase autophosphorylation and downstream signalling molecules

To determine whether C-18 inhibits ligand-induced Tie2 phosphorylation, we exposed HUVEC to Ang-1, immunoprecipitated the Tie2 receptor and blotted the precipitates with a phosphotyrosine antibody (Figure 5a). As expected, Ang-1 promoted Tie2 tyrosine phosphorylation, but concentrations of C-18 up to 10 μM did not block the action of Ang-1, suggesting that C-18 is not a Tie2 inhibitor as previously suggested by *in vitro* kinase assays. Similarly to what was observed for the Tie2 receptor, C-18 was unable to inhibit phosphorylation of VEGR2 on Tyr1175 triggered by VEGF, suggesting that C-18 does not inhibit the activity of this RTK either (Figure 5b).

Inhibition of angiogenic signalling by C-18

Ang-1 and VEGF use many common signalling cascades including phosphoinositide-3 kinase/Akt and MAP kinase pathways. To investigate whether C-18 inhibits the above-mentioned angiogenesis-related cascades, we pretreated cells with C-18 and used phospho-specific antibodies to probe the activation status. Both VEGF and Ang-1 increased the phosphorylation of residues critical for activation of ERK1/2, p38 and Akt (Figure 6). In agreement with its ability to inhibit biological responses to VEGF and Ang-1, C-18 blocked the effects of both growth factors on ERK1/2, p38 and Akt activation.

In vivo *anti-angiogenic properties of C-18*

To investigate if C-18 exhibits anti-angiogenic properties *in vivo*, we tested this compound in the CAM model. Different doses of C-18 were applied in restricted areas of the chorioallantoic membranes of fertilized eggs, and the length of the vascular network was assessed using image analysis software (Figure 7). Treatment with increasing concentrations of C-18 resulted in a dose-dependent reduction of vessel length, reaching approximately a 50% reduction at 100 pmol per egg, making C-18 one of the most effective inhibitors in this system tested in our laboratory.

Discussion

We have previously demonstrated that sulindac analogues inhibit angiogenesis-relevant RTKs (Gourzoulidou *et al*.,

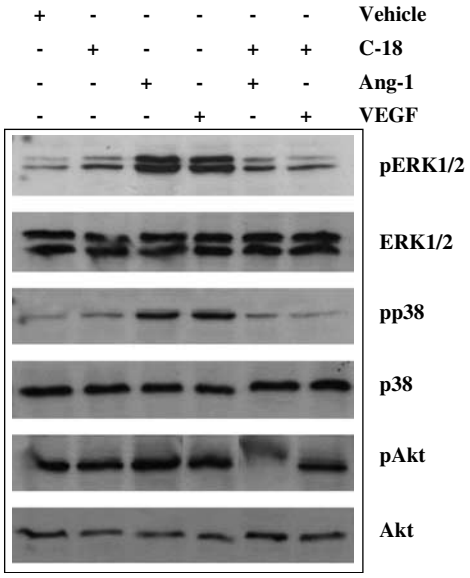

Figure 6 C-18 inhibits Ang-1- and vascular endothelial growth factor (VEGF)-induced mitogen-activated protein kinase (MAPK) and Akt activation. Serum-starved human umbilical vein endothelial cells (HUVECs) were pretreated with C-18 (1 nM) for 15 min and incubated with Ang-1 (250 ng ml^{-1}) or VEGF (50 ng ml^{-1}) for an additional 20 min. Consequently, cells were lysed and samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting using antibodies that specifically recognize the phosphorylated or the total forms of the kinases. Blots shown are representative of experiments repeated at least twice with similar results. Ang, angiopoietin; C-18, 2-((1E,Z)-1-benzylidene-5-bromo-2-methyl-1H-inden-3-yl)acetic acid.

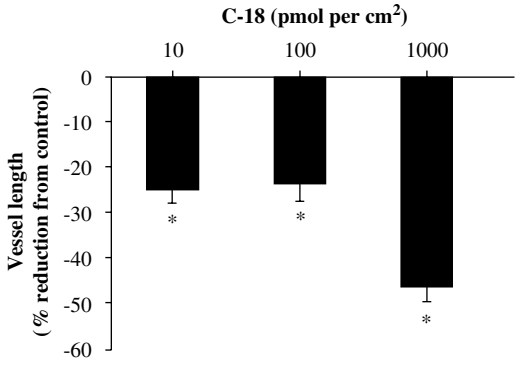

Figure 7 C-18 inhibits neovessel formation *in vivo*. C-18 or vehicle (dimethylsulphoxide (DMSO) in phosphate-buffered saline) was added to restricted areas of the chorioallantoic membrane (CAM) of fertilized eggs, as described in Materials and methods. After 48 h, CAMs were fixed and vessel length evaluated using image analysis software. Data are expressed as means ± s.e.mean; $n = 30$–50 eggs per group; *$P < 0.05$ vs control. C-18, 2-((1E,Z)-1-benzylidene-5-bromo-2-methyl-1H-inden-3-yl)acetic acid.

2005). Among the analogues tested, a compound, which we refer to as C-18, inhibited RTK Tie2 *in vitro*.

In the present study, this putative Tie2 inhibitor abolished Ang-1 stimulated HUVEC migration at 100 pM, at a concentration well below its reported IC_{50} for Tie2 activity inhibition (1 μM) (Gourzoulidou *et al*., 2005), suggesting that C-18 is either actively taken up by cells leading to intracellular accumulation of this drug, or C-18 inhibits another target(s) necessary for migration at lower concentrations than the one needed to inhibit Tie2. To directly test the ability of C-18 to block activation of Tie2 by its native ligand, HUVECs were exposed to Ang-1, and tyrosine phosphorylation was studied in Tie2 immunoprecipitates. Phosphorylation of Tyr-992, -1002 and -1008 (human sequence) is known to be crucial for Ang-1-stimulated signalling and actions (Ward and Dumont, 2002). Indeed, incubation of HUVEC with Ang-1 promoted Tie2 phosphotyrosine content; however, C-18 when used at 1 nM or 10 μM failed to block the action of Ang-1. This finding is in conflict with our previous report that C-18 inhibits Tie2 activity in *in vitro* kinase assays (Gourzoulidou *et al*., 2005) and can be explained by the fact that (1) during the *in vitro* kinase assays, we utilized a recombinant truncated form of the receptor and (2) basal (not agonist-stimulated) activity of the Tie2 was determined.

Serum-deprivation of HUVEC triggers apoptosis that can be monitored by measuring caspase-3 activity. In line with previous reports, Ang-1 reduced the increase in caspase-3 activity brought about by serum withdrawal (Harfouche *et al*., 2002); however, C-18 did not reverse the effect of Ang-1 on caspase-3 activity. The lack of effect of C-18 at the receptor level is in agreement with the finding that C-18 does not block the antiapoptotic action of Ang-1.

To better characterize the effects of C-18 on migration, we determined the ability of this sulindac analogue to inhibit migration in response to VEGF and FGF-2. Indeed, pretreatment with C-18 abolished the migratory properties to both growth factors, suggesting that this agent inhibits a common, RTK-stimulated, signalling pathway involved in cell motility. To determine the level at which C-18 exerts its inhibitory effects, we concentrated on VEGF-stimulated migration and signalling. Cells were exposed to VEGF with or without C-18 pretreatment and the phosphorylation status of Tyr-1175 of VEGFR2 was determined, as this residue is critical in mediating migratory responses (Ferrara *et al*., 2003). VEGFR2 phosphorylation was induced by its cognate ligand, and this response was not modified by C-18, suggesting that C-18 acts downstream of VEGFR2 to block cell motility. We and others have previously shown that VEGF-stimulated migration depends on NO/cGMP signalling, as NO synthase and sGC inhibitors block VEGF-induced motility (Ziche *et al*., 1997; Pyriochou *et al*., 2006). Pretreatment with C-18 did not alter the migratory response to the sGC activator BAY 41-2272, indicating that C-18 targets a molecule(s) downstream of VEGFR2, but upstream of sGC.

To further study the mechanism of C-18 action, we incubated cells with either Ang-1 or VEGF and tested the ability of the sulindac analogue to inhibit activation of cascades relevant to angiogenesis. In line with previous reports, both growth factors stimulated ERK1/2 and p38 mitogen-activated protein kinase (MAPK) phosphorylation, as well as Akt activation (Jones *et al*., 2001; Harfouche *et al*., 2003; Tsigkos *et al*., 2003). C-18 inhibited activation of all three pathways tested. In agreement with our results, other non-steroidal anti-inflammatory drugs including indomethacin and sulindac metabolites at high micromolar concentrations have been show to inhibit ERK1/2 and p38 activation (Jones *et al*., 1999; Rice *et al*., 2001).

Having established that C-18 blocks growth factor-induced migration, we next sought to determine its effects on other angiogenesis-related properties of endothelial cells, namely growth and tube-like structure formation. Most agents that inhibit angiogenic responses also block endothelial cell proliferation; indeed, C-18 blocked serum-induced endothelial proliferation. However, unexpectedly for an angiogenesis inhibitor, the sulindac analogue stimulated tube-like structure formation on matrigel. To evaluate the *in vivo* properties of this compound, we tested C-18 in the CAM model. C-18 exerted a strong anti-angiogenic activity, reaching a 50% decrease in vascular length, making this compound one of the most potent and effective used in our system. The differential effect of C-18 on capillary morphogenesis *in vitro* (matrigel) vs *in vivo* (CAM) can be easily explained, as matrigel-stimulated tube-like network formation on matrigel is regulated by extracellular matrix and involves only endothelial cells, while neovascularization in the CAM depends on multiple soluble and matrix-derived stimuli and involves more cell types. It should also be noted that, sulindac sulphone, an oxidized sulindac analogue that does not inhibit cyclo-oxygenase, was also reported to block angiogenesis in the CAM (Elwich-Flis *et al*., 2003).

In summary, our findings suggest that C-18 is not a direct Tie2 or VEGFR2 inhibitor *in vivo*, but expresses antimitogenic and anti-migratory properties against key angiogenic growth factors. The observations that C-18 is capable of inhibiting activation of ERK1/2, p38 and Akt suggest that this sulindac analogue interferes with signalling molecule(s) downstream of RTK, but upstream of MAPK. This observation is in line with the finding that some sulindac analogues inhibit Ras-regulated pathways (Muller *et al*., 2004; Waldmann *et al*., 2004). Although the exact mechanism of action of C-18 remains elusive, we have shown that this agent is a potent inhibitor of angiogenesis *in vivo*, suggesting that sulindac analogues could be investigated for their usefulness in anti-angiogenesis therapies.

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Conflict of interest

The authors state no conflict of interest.

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